Live-cell imaging reveals the spatiotemporal organization of endogenous RNA polymerase II phosphorylation at a single gene

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The carboxyl-terminal domain of RNA polymerase II (RNAP2) is phosphorylated during transcription in eukaryotic cells. While residue-specific phosphorylation has been mapped with exquisite spatial resolution along the 1D genome in a population of fixed cells using immunoprecipitation-based assays, the timing, kinetics, and spatial organization of phosphorylation along a single-copy gene have not yet been measured in living cells. Here, we achieve this by combining multi-color, single-molecule microscopy with fluorescent antibody-based probes that specifically bind to different phosphorylated forms of endogenous RNAP2 in living cells. Applying this methodology to a single-copy HIV-1 reporter gene provides live-cell evidence for heterogeneity in the distribution of RNAP2 along the length of the gene as well as Serine 5 phosphorylated RNAP2 clusters that remain separated in both space and time from nascent mRNA synthesis. Computational models determine that 5 to 40 RNAP2 cluster around the promoter during a typical transcriptional burst, with most phosphorylated at Serine 5 within 6 seconds of arrival and roughly half escaping the promoter in ~1.5 minutes. Taken together, our data provide live-cell support for the notion of efficient transcription clusters that transiently form around promoters and contain high concentrations of RNAP2 phosphorylated at Serine 5.
n eukaryotic cells, the catalytic RPB1 subunit of RNA polymerase II (RNAP2) possesses an extended carboxy-terminal domain (CTD) that consists of heptapeptide repeats (52 in humans) with a consensus sequence (Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7). The CTD region is dynamically phosphorylated as RNAP2 progresses through the transcription cycle, regulating each step of transcription, from initiation to termination. In some models, RNAP2 is recruited to promoters in an unphosphorylated form (CTD-RNAP2), but is later phosphorylated at Serine 5 (Ser5ph-RNAP2) upon initiation and at Serine 2 (Ser2ph-RNAP2) during active elongation1–4. Interest in the CTD has recently increased due to observations of highly dynamic RNAP2 clustering8,9 that correlates with the phosphorylation status of the CTD7,8. In particular, recent data suggest that a transcriptional cluster forms around gene promoters early in the transcription cycle. The cluster is thought to be enriched in unphosphorylated- and Ser5ph-RNAP2 that appear to constrain chromatin movement near the transcription start site9. However, upon transcriptional activation, hyperphosphorylation of RNAP2 at Ser2 allows the enzyme to escape the cluster and begin active elongation7,9. The dynamic clustering of RNAP2 involves many steps and a complex orchestration of multiple factors and could therefore represent a global form of transcriptional regulation10.

RNAP2 phosphorylation throughout the transcription cycle has traditionally been studied in fixed cells using immunoprecipitation-based assays1,2,3,11. These studies provide precise spatial maps of the average positions of RNAP2 along the 1D genome. Unfortunately, the inherent averaging masks heterogeneity, and the procedure limits temporal resolution to timescales of tens of minutes or longer12. RNAP2 dynamics can instead be imaged and quantified in living cells using fluorescence microscopy, overcoming the limitations of traditional assays. Recent single-molecule tracking technologies13–17 has made it possible to monitor single RNAP2 as they bind at non-specific locations throughout the genome, as well as a specific, single-copy genes6,17 pre-marked with MS219,20 or PP721 RNA stem–loops (that are lit up co-transcriptionally when, respectively, fluorescent MS2 or PP7 coat proteins bind to them). Each of these studies used permanent fluorescent fusion tags to track RNAP2. Fusion tags are incapable of discerning post-translational modifications to RNAP2, including transcription cycle-associated phosphorylation events.

One way to resolve post-translational modifications to RNAP2 is to use antibody-based probes that bind and light up specific modifications to residues within the CTD in vivo15–26. However, the signal-to-noise is limited with this approach because of the presence of unbound and freely diffusing probes that increase the fluorescence background (BG). Applications have therefore been restricted to large tandem gene arrays. Signal-to-noise is amplified by the multiple copies of a gene within these arrays, but heterogeneity from one gene copy to another is again masked by averaging27. Therefore, the spatiotemporal dynamics of RNAP2 phosphorylation at single-copy genes remain unclear.

Here, we combine multicolor single-molecule microscopy, complementary fluorescent antibody-based probes, and rigorous computational modeling to visualize, quantify, and predict endogenous RNAP2 phosphorylation dynamics at a single-copy reporter gene in living cells. This unique combination of technologies allows us to directly visualize the temporal ordering and spatial organization of RNAP2 phosphorylation and mRNA synthesis throughout the transcription cycle at the reporter gene. We find evidence for relatively high concentrations of RNAP2 near the beginning vs. end of the gene that are both spatially and temporally separate from elongating RNAP2 and nascent mRNA synthesis. Collectively, our data provide live-cell support for the existence of higher-order, phosphorylation-dependent transcriptional clusters that dynamically form and surround active genes throughout the transcription cycle.

Results
Technology to visualize endogenous RNAP2 transcription cycle dynamics at a single gene. To visualize the spatiotemporal dynamics of endogenous RNAP2 phosphorylation at a single gene, we used an established HeLa cell line (H-128) harboring an MS2-tagged HIV-1 reporter gene and stably expressing both GFP-tagged MS2 coat protein (MCP) and an untagged HIV-1 trans-activator of transcription (Tat)20. We chose HIV-1 as our reporter gene because it is a prototypical model for RNAP2 phosphorylation24. The HIV-1 reporter is strongly active in our cell line due to persistent stimulation by Tat, producing a bright MCP signal that pinpoints the location of the transcription site (TS) and gauges its activity in real-time25 (Fig. 1a). Consistent with this strong signal, immunostaining experiments in fixed cells revealed the TS is highly enriched in RNAP2 and relatively depleted of histones and their epigenetic modifications (Supplementary Fig. 1a, b). Chromatin immunoprecipitation (ChIP) experiments further confirmed the presence of CTD-RNAP2 and its phosphorylated forms Ser5ph- and Ser2ph-RNAP2, respectively. In particular, we detected that CTD-RNAP2 and Ser5ph-RNAP2 signals are highest at the transcription start site, whereas Ser2ph-RNAP2 is highest towards the end of the gene (Fig. 1b). However, because these data come from a population of fixed cells, whether the various forms of RNAP2 are present at the same time and place and whether or not they appear in a preferred order is difficult to extract from this assay.

To better characterize the spatiotemporal dynamics of single-cell RNAP2 modifications during transcription, we loaded fluorescent fragmented antibodies (Fab, generated from the same antibodies used in ChIP22,29 recognizing (1) the CTD of RNAP2 (anti-CTD RNAP2) without or with residue-specific phosphorylations, and (2) heptad repeats within the CTD that are phosphorylated at Serine 5 (anti-Ser5ph RNAP2). These antibodies have previously been shown to be specific for their respective targets via Western blotting and ELISA27 and in ChIP-seq30 experiments. Fab generated from these antibodies has also been shown to rapidly bind and unbind their targets, making them valuable for monitoring temporal changes in RNAP2 phosphorylation27. Consistent with anti-CTD Fab labeling all RNAP2 and anti-Ser5ph Fab labeling a subset of RNAP2, we observed regions within the nucleus with RNAP2 enriched or depleted with Ser5ph (Supplementary Fig. 2). These capabilities allowed us to distinguish three distinct steps of the transcription cycle at the HIV-1 reporter gene: RNAP2 recruitment (marked by Fab against CTD-RNAP2), initiation (marked by both Fab against CTD-RNAP2 and Fab against Ser5ph-RNAP2), and elongation (marked by all Fab and MCP binding to mRNA), as depicted in Fig. 1a, c. Although we attempted to also visualize Ser2ph at the locus with our Fab, signal-to-noise was insufficient to detect in living cells, presumably because the antibody is not sensitive enough to recognize this modification at the single-gene level.

Nevertheless, this setup has several advantages that collectively enhance signal-to-noise at the TS. First, Fab binds endogenous RNAP2, so all RNAP2 in the cell has a high likelihood to be labeled without having to genetically engineer a fusion knock-in tag8,31 and/or alpha-amanatin resistance5. Second, fluorescence is naturally amplified since mammalian RNAP2 contains 52 heptad repeats in its CTD32, each of which can be bound by a fluorescent Fab at the TS. Third, Fab continually binds and unbinds RNAP2, mitigating the loss of fluorescence due to local photobleaching. In combination with a multi-color, single-
Fig. 1 A system for imaging the endogenous RNAP2 transcription cycle at single genes. a Schematic of the system. The reporter gene is controlled by the HIV-1 promoter and is tagged with a 128xMS2 cassette (blue bar). RNAP2 is represented in gray. RNA is marked by MCP-GFP that binds to the transcribed MS2 stem-loops (mRNA, blue). The recruited and initiated RNAP2 are labeled by Fabs (conjugated with CF640 and Cy3) that bind unphosphorylated and phosphorylated CTD RNAP2 heptad repeats (CTD, red) and Serine 5 phosphorylated repeats (Ser5ph, green), respectively. b Average chromatin immunoprecipitation occupancy of CTD-RNAP2 (red, upper panel), Ser5ph-RNAP2 (green, middle panel), and Ser2ph-RNAP2 (blue, lower panel) across the HIV-1 reporter gene (positions 1–10 are highlighted in the cartoon above). Data are presented as mean values ± S.E.M. c Sample live-cell showing CTD-RNAP2, Ser5ph-RNAP2, and mRNA co-localizing at the transcription site (TS), n = 9 cells in 4 independent experiments. d Normalized intensity at the TS over time from the cell in (c) for CTD-RNAP2 (red circles), Ser5ph-RNAP2 (green squares), and mRNA (blue diamonds).
molecule microscope\textsuperscript{33} employing oblique HILO illumination to enhance signal-to-noise by an order of magnitude\textsuperscript{13}; these advantages allowed us to generate movies in which we monitored endogenous RNAP2 phosphorylation dynamics at the HIV-1 reporter gene in 3-colors.

As shown in Fig. 1c, d, movies revealed correlated fluctuations between the mRNA signal and endogenous CTD-RNAP2 and Ser5ph-RNAP2 signals at the TS. To ensure correlations were not an artifact of focusing issues, we tracked the TS in 3D (by imaging 13 z-planes per time point) to keep the MS2 signal continually in focus (Supplementary Fig. 1c, left panel). The correlations were also not caused by photobleaching, as signals fluctuated both up and down throughout the entire imaging time course, remaining on average constant (Supplementary Fig. 1c). Finally, to rule out the possibility that correlated fluctuations were caused by bleed-through from one fluorescence channel to another, we re-imaged cells lacking Fab. In all cases, no bleed-through was observed (Supplementary Fig. 1d, e), as quantified by the covariance between channels (Supplementary Fig. 1f). We, therefore, conclude the correlations reflect natural bursts in endogenous transcriptional activity at the HIV-1 reporter gene, demonstrating our ability to detect and quantify endogenous RNAP2 phosphorylation dynamics at a single-copy gene.

Long-term imaging of fluctuations at the reporter gene reveals temporal ordering of RNAP2 phosphorylation. In the majority of cells, the mRNA was steadily produced by the HIV-1 reporter gene, with strong signals persisting for hours at a time. In a few cells, the mRNA signal completely disappeared, indicating a loss of nearly all transcription activity. We were interested in capturing these rare events in a single time course to better discriminate the relative timing of our RNAP2 and mRNA signals. To accomplish this, we adjusted our imaging conditions to optimize detection of all three signals in single cells over a period of three hours (200-time points), as exemplified in Fig. 2a–c (also see Supplementary Movie 1, and Supplementary Fig. 3a–c). We again imaged in z-stacks (13 planes spaced by 0.5 μm) covering the whole nucleus at each time point throughout the entire experiment. We were therefore confident that the fluctuations were due to changes in transcription activity and not related to transcription site movement into and out of the focal plane (Supplementary Fig. 3d). With these imaging conditions, we found cells in which the mRNA signal turned on and off up to four times, indicating bursts of transcription and multiple complete transcription cycles. Consistent with our previous result, signals at the transcription site were highly correlated and fluctuated generally in unison, although there were distinct periods of time when one signal could be seen for multiple frames in the absence of some other signals. This again ruled out bleed-through and suggested the signals were not perfectly synchronized. To ensure the correlated fluctuations were specific to the locus and not cell-wide, we verified that covariances between the mRNA signal and the CTD or Ser5ph signals were significantly stronger when both signals were measured at the transcription site compared to when one or both signals were measured a short distance (p1) from the transcription site (Supplementary Fig. 3e–g).

Having established a well-controlled system to examine fluctuations at a single gene, we were confident in our ability to quantify the temporal ordering of RNAP2 and mRNA throughout the transcription cycle. One thing that stood out was that peaks and troughs in the mRNA signal tended to come after the peaks and troughs in the RNAP2 signals. Although there were some exceptions due to the stochasticity of the system, in some cells this behavior was seen multiple times in even single time series (for example, see valleys at t_{1-4} = 16, 75, 94, and 113 min in Fig. 2b, c). To better quantify this effect, we selected all events at which the mRNA signal dropped below a threshold value, extracted all three signal channels from seven minutes before to seven minutes after each event, and aligned all signals relative to these mRNA minima event times (Fig. 2d). This analysis revealed two important aspects of the dynamics of our system. First, the analysis confirmed the signals were strongly correlated since strong minima could be observed in all channels. These minima were significant compared to the results from unaligned signals (gray diamonds in Fig. 2d, p value of $1.72 \times 10^{-10}$ for Ser5ph- and $6.39 \times 10^{-4}$ for CTD-RNAP2). Such strong correlation between mRNA production at the HIV-1 reporter and endogenous RNAP2 would suggest the reporter is not part of a larger transcriptional unit containing multiple genes. Second, the analysis indicated a temporal ordering, with both RNAP2 signals coming before mRNA by 0.96 ± 0.55 min for CTD-RNAP2 (p value $3.65 \times 10^{-3}$) and 0.88 ± 0.24 min for Ser5ph-RNAP2 (p value $1.28 \times 10^{-3}$). This delay makes sense because RNAP2 must escape the promoter and elongate 0.7 kb before it reaches the MS2 repeats. The CTD-RNAP2 signal also slightly preceded the Ser5ph-RNAP2 signal, although the delay was not significant at our sampling rate. This suggests nearly all RNAP2 at the locus either come in pre-phosphorylated or are rapidly phosphorylated at Serine 5 within a minute of arrival.

Spatial organization of CTD phosphorylation at the reporter gene. RNAP2 is thought to be organized in phosphorylation-dependent clusters\textsuperscript{7,4}. To test this hypothesis, we measured the center position in X and Y of CTD-RNAP2, Ser5ph-RNAP2, and mRNA at the reporter gene over time (Fig. 2e–g). If the hypothesis is correct, we would expect to see some spatial separation in our different RNAP2 and mRNA signals. To confirm this hypothesis, we calculated the Euclidean distance between each pair of signals. As Fig. 2g illustrates, the distances between signals changed over time but were spatially organized such that the RNAP2 signals were significantly separated from mRNA.

Although there was considerable variation from cell to cell, this trend could be seen in the median positions from the whole population of transcription sites we tracked (Fig. 2h). Specifically, the median distance from mRNA to CTD-RNAP2 was ~181 nm compared to ~148 nm for Ser5ph-RNAP2 (p value 0.032). Likewise, the median distance between the two forms of RNAP2 was just ~93 nm, significantly smaller than between either form of RNAP2 and mRNA (p value < 6.97 x 10^{-11}) (Fig. 2h and Supplementary Fig. 3h). This spatial separation was consistent across the cells we analyzed (Supplementary Fig. 4) and independent of the strength of transcription as gauged by CTD-RNAP2, Ser5ph-RNAP2, and mRNA signal intensities. Together, these results demonstrate that RNAP2 is spatially organized within the transcription site, with active mRNA synthesis spatially distinct from clusters of CTD-RNAP2 and Ser5ph-RNAP2.

Fluctuation dynamics and statistics are captured by a simple model of transcription bursting. We wanted to obtain a more universal picture of RNAP2 phosphorylation dynamics at the HIV-1 reporter gene. We therefore performed correlation analysis\textsuperscript{21,34,35} using all time points in all time series, similar to fluorescence correlation spectroscopy\textsuperscript{36}. This technique is ideal for extracting information from noisy data provided there are a sufficient number of time series and/or time points. We began with an auto-correlation analysis, to see how long each signal remains correlated with itself given a lag time (r) (Fig. 3a). The auto-correlation of each signal decays with increasing lag time...
and eventually flattens out near zero. We define the dwell time as the lag time at which the auto-correlation falls below 20% of its initial zero-lag value. According to this analysis, the two forms of RNAP2 had shorter average dwell times than mRNA, indicating RNAP2 was often unsuccessful in reaching the end of the gene and synthesizing an mRNA.

Next, we calculated the cross-correlation between signals. Consistent with our previous analysis aligning local minima, all possible pairs of signals were strongly correlated, as seen by large peaks in the cross-correlation curves near $\tau = 0$ (Fig. 3b). Measuring the precise position of each peak revealed the mRNA signal came substantially later than the CTD-RNAP2 and Ser5ph-RNAP2 signals, while the CTD-RNAP2 and Ser5ph-RNAP2 signals appeared at roughly the same time (within the 1 min sampling time of experiments). To better resolve the time delay between the CTD-RNAP2 and Ser5ph-RNAP2 signals, we re-imaged the HIV-1 TS in a single plane at a much faster frame rate (150 msec/frame) for a total of 1000 time points (150 sec). Although these higher temporal resolution experiments are much too short to capture the full auto- and cross-correlation curves,
Fig. 2 Spatiotemporal organization of the RNAP2 CTD cycle at the HIV-1 reporter gene. a Sample cell showing co-localization of CTD-RNAP2, Ser5ph-RNAP2, and mRNA signals at the transcription site (TS), n = 13 out of 20 cells. b The TS from (a) at select times. c Normalized intensity fluctuations at the TS for CTD-RNAP2 (red circles), Ser5ph-RNAP2 (green squares), and mRNA (blue diamonds) vs. time. Times of minimal mRNA (less than 0.20 arb.units) are marked with dashed gray lines (ħ...). d The average normalized intensity of each signal surrounding times of minimal mRNA. Both the Ser5ph-RNAP2 and CTD-RNAP2 signals have deep minima well below the steady-state value (dashed horizontal line). Solid connecting lines show the Gaussian fit, and solid vertical lines mark the minima with a lighter shadow depicting the S.E.M from the Gaussian

- They are sufficient to resolve the short time-lag dynamics (Supplementary Fig. 5), and they revealed cross-correlation asymmetry with an off-center peak indicating that the Serph-RNAP2 signal comes roughly 3–6 sec after the CTD-RNAP2 signal. The various delays we measure are consistent with the temporal ordering we saw by aligning local minima of the mRNA signal (Fig. 2d) and provide further evidence that RNAP2 phosphorylation at Serine 5 is very rapid at the transcription site.

- We next sought to find a quantitative model to unify our diverse data sets (Fig. 3c). We required that our model must simultaneously fit all three auto-correlation curves (Fig. 3a) and all three cross-correlation curves (Fig. 3b). To further constrain the model, we also counted mRNA at transcription sites by comparing their intensities to single mature mRNAs using FISH--quant (Fig. 3d, bottom). Consistent with an earlier report, we found the HIV-1 reporter contained an average of μ = 15.5 mRNA with a relatively large standard deviation of σ = 10.55 and Fano Factor of σ²/μ = 7.1.

- To unify our data, we posed several models with different levels of complexity (Supplementary Fig. 6). Each model considered a promoter with bursty expression. This was represented by specifying distinct active (ON) and inactive (OFF) promoter states with OFF-to-ON and ON-to-OFF transitions rates k_on and k_off, respectively. When the promoter is ON, RNAP2 is recruited at a rate k recruiting. Upon fitting these models to our data, the fitted burst duration was much shorter than the 1-min experimental sampling time (i.e., k off < 1 min). This allowed us to simplify the model to one with burst frequency ω = 1/(k_on + 1/k off) and geometrically distributed bursts with average size β = k / k off. In all models that fit our data, RNAP2 could unsuccessfully depart the promoter at rate k off or escape at rate k esc. After the escape, the RNAP2 would complete transcription at a combined rate k_t that includes both elongation and processing.

- In the minimal model that matched all data, CTD-RNAP2 were immediately phosphorylated upon arrival at the promoter, which was consistent with the rapid (<1 min) Serine 5 phosphorylation we observed (Supplementary Fig. 5). We also explored several more complicated models with separate steps for initiation, elongation, and processing, post-transcriptional mRNA retention, or with separate events describing Serine phosphorylation/initiation and de-phosphorylation/abortion (Supplementary Fig. 6). Each model was fit separately to maximize the likelihood for all observed data, but the inclusion of additional mechanisms and free parameters provided only marginal improvements to the overall fit and resulted in much larger parameter uncertainties. Therefore, we used the Bayesian Information Criteria (BIC) to select our final model as the best choice given our available data (See tables in Supplementary Fig. 6).

- By simultaneously fitting all six correlation plots (Fig. 3a, b) as well as the nascent mRNA means and variances, we could estimate the best model’s five parameters with excellent precision (Supplementary Fig. 7). The best-fit parameter values and their uncertainties are provided in Fig. 3e. According to the best fit, bursts of RNAP2 occur on average every 1/ω ≈ 2.3 min and have an average size of about β ≈ 15 molecules per burst. Of the RNAP2 that arrive at the promoter, a substantial fraction f = k esc / (k esc + k off) = 0.46 escape the promoter and complete transcription, leading to convos of about f · β = ~7 RNAP2 per burst. Each mRNA takes an average of 1/k_t = 5 min to complete elongation and processing, meaning that on average the HIV-1 reporter contains mRNA originating from ω / k_t = ~2 consecutive bursts. Overall, the model predicts that there is an average of ~20 RNAP2 on the gene in steady-state, with an average of ~5 in the cluster near the promoter in an unphosphorylated or Ser5ph form, and ~15 elongating or processing near the end of the gene (see Table 1). This average picture is somewhat misleading, however, as the number of RNAP2 within the cluster fluctuates dramatically due to randomly timed bursts. According to our simulations, there are periods when as many as ~90 RNAP2 come in at a time interspersed by brief and random silent periods of low RNAP2 occupancy (Supplementary Fig. 8a).

- After fitting the model to capture the auto- and cross-correlation functions and the mean and variance of the mRNA distribution, we verified that it also correctly predicted the full probability distributions for the number of nascent mRNA molecules and RNAP2 signal intensities at the HIV-1 transcription site (Fig. 3d). We also simulated normalized intensities including shot noise (Fig. 3f), and these look similar to our measured trajectories (Fig. 2c). The shot noise was estimated directly from the experiments by comparing the observed zero-lag covariance G(0) compared to an estimate for the zero-lag autocovariance found by interpolation from the short, but nonzero time lags. These shot noise standard deviations were found to be 1.98×, 1.42×, and 0.41× of the standard deviation for CTD, Ser5ph, and MS2 signals, respectively. Finally, we simulated ChIP data for our single-gene reporter (Supplementary Fig. 8b, c). To do this, we assumed an elongation rate of 4.1 kb/min (measured previously at this locus by analyzing the MS2 stochastic fluorescence fluctuations and processing rate of 0.27 min−1 (so elongation and processing times sum to our fitted 1/k_t completion time). With these rates, the CTD/Ser5ph-RNAP2 simulated ChIP signals from active genes displayed strong peaks at the beginning and end of the gene, as we observed in Fig. 1b (compare to Supplementary Fig. 8b, c). Overall, the excellent match between data and simulations indicates our best-fit model faithfully captures transcription dynamics at the HIV-1 reporter. To facilitate further exploration of our model, we provide a graphical user interface (GUI) [https://doi.org/10.5281/zenodo.4631141]. The GUI allows...
exploration of how each model parameter affects model predictions, including trajectories, auto- and cross-correlations, distributions of spot intensities, simulated ChIP data, and several derived quantities to describe the CTD-RNAP2, Ser5ph-RNAP2, and mRNA burst dynamics (Supplementary Fig. 9).

Inhibiting distinct steps of the transcription cycle provides further evidence for the spatiotemporal organization of RNAP2 phosphorylation. So far, our collective data and modeling suggest a precise temporal ordering of transcription dynamics, beginning with the recruitment of CTD-RNAP2,
followed by rapid initiation in 3–6 s (indicated by Ser5ph-RNAP2), and promoter escape and elongation within another minute or so (indicated by mRNA). Our data also provide evidence of heterogeneity in the distribution of RNAP2 along the gene, with high concentrations near the beginning and end of the gene (Fig. 1b). To further test our system, we perturbed it by adding three different transcription inhibitors: Triptolide (TPL), THZ1, and Flavopiridol (Flav) (Fig. 4). We began by inhibiting the earliest steps in the transcription cycle to attempt to prevent the formation of the RNAP2 cluster. To achieve this we added TPL, a small-molecule inhibitor that prevents promoter DNA opening and transcription initiation by inhibiting the DNA-dependent ATPase activity of the XBP subunit of TFIIH.4,41 TPL has also been shown to induce RNAP2 degradation on the hours timescale42, so we imaged for just 30 consecutive minutes to focus on the more immediate impact of TFIIH inhibition. The addition of 5 μM TPL led to a rapid and dramatic loss of both mRNA and all RNAP2 signals at the TS within just ~10 min (Fig. 4a–d, and Supplementary Movie 2). Consistent with our previous findings, we observed a temporal ordering in the TPL-induced run-off of RNA2 (Fig. 4c), with CTD-RNAP2 signals dropping earlier than Ser5ph-RNAP2, followed by mRNA. This ordering was observed in seven out of ten single cells we measured. Of these, four exhibited clear separation between the three traces (inset in Fig. 4c). Since steps that are later in the CTD cycle necessarily take longer to respond to drugs, this ordering provides further evidence that CTD-RNAP2 slightly precedes Ser5ph-RNAP2 by less than a minute, and that both RNAP2 signals come significantly earlier than mRNA. These data also demonstrate that the opening of promoter DNA by XBP is a requirement for the formation of RNAP2 clusters. This can work by at least two mechanisms: (1) All the Ser5ph-RNAP2 underwent initiation and abortion, but RNAP2 kept its Serine 5 phosphorylation; (2) Initiation of the first RNAP2 activates CDK7, which can phosphorylate many RNAP2 within the cluster.

We next used THZ1, which inhibits RNAP2 CTD phosphorylation at Serine 5 by targeting the TFIIH kinase CDK7, thereby preventing promoter pausing, mRNA capping, and productive elongation1,18,43. In contrast to TPL, THZ1 has a slower action, so a higher concentration and longer exposure to this drug were needed to see an effect in real-time. Treatment with 15 μM THZ1 led to a reduction in the mRNA signal at the HIV-1 reporter within 25 min (Fig. 4c). Likewise, both CTD-RNAP2 and Ser5ph-RNAP2 levels were on average reduced. Interestingly, in some single cells, we observed large, temporally ordered bursts in the levels of CTD-RNAP2 and Ser5ph-RNAP2, despite continued inhibition and overall loss of mRNA. These large bursts could even achieve RNAP2 levels that were as high as pretreatment levels (the thicker black curve in Fig. 4c highlights one example). Presumably, these bursts occur because there is residual TFIIH left in the cell that are not yet inhibited by THZ1, or because recently aborted RNAP2 retain their Ser5ph within the cluster. Since mRNA levels did not burst to the same degree, we conclude the bursts arise from clusters of RNAP2 near the promoter that initiate but fail to escape. These transient clusters near the beginning of the gene are consistent with the high concentration of RNAP2 near the promoter we observed by ChIP (Fig. 1b) and are also consistent with the ChIP predictions of our best-fit model (Supplementary Fig. 8b, c).

We next blocked a later step in the transcription cycle using 1 μM Flav, a drug that prevents transcription elongation and RNAP2 CTD phosphorylation at Serine 2 by inhibiting the CDK9 activity of P-TEFb18,44. Like THZ1, Flav also reduced the intensity of the mRNA signal, this time within ~15 min (Fig. 4f). However, CTD-RNAP2 and Ser5ph-RNAP2 signals remained relatively unchanged, exhibiting large fluctuations and a slight overall reduction on average. This difference from THZ1 can be attributed to the later action of Flav in the transcription cycle. The high levels of CTD-RNAP2 and Ser5ph-RNAP2 signals that remained post-Flav again support a dynamic clustering model4,5,7–9 in which most RNAP2 are already phosphorylated at Serine 5 and presumably make repeated attempts at initiation and promoter escape.

Finally, we attempted to qualitatively recapitulate these perturbations using our best-fit model. To do so, we evaluated several hypothetical mechanisms in which transcription is inhibited by reducing one or more of the rates, including burst statistics (ω or β), the promoter escape rate kesc, or the completion rate kβ. According to simulations, inhibiting earlier steps (ω or β) in the transcription cycle led to the sequential loss of all RNAP2 and mRNA signals at the transcription site at a rate governed by the time scale of mRNA elongation and processing (Supplementary Fig. 10a), reminiscent of our TPL experiments. In contrast, inhibiting a later step (kesc), led to a retention of large numbers of RNAP2 in the cluster that undergoes relatively large and rapidly changing fluctuations (Supplementary Fig. 10b), reminiscent of our THZ1 experiments. Blocking (kesc) and reducing kβ by 30% led to a slight reduction in the mRNA signal and even less decrease in the RNAP2 signals with relatively large fluctuations (Supplementary Fig. 10c), reminiscent of our Flav experiments. We also blocked bursts (either ω or β) and reduced kβ by 30% and obtained an overall reduction of all the signals (Supplementary Fig. 10d) that do not represent any of the inhibitors tested here. The similarity between these simulations and our experimental perturbations provides further support for our model and also provides evidence that the tested inhibitors act on distinct stages of the RNAP2 transcription cycle.

### Table 1: Derived quantities and confidence intervals resulting from the model fit to data.

| Derived quantity | Formula | Value (95% CI range) |
|------------------|---------|----------------------|
| Average RNAP2 burst size | β | 15.4 (11.76-61.83) |
| Average RNAP2 burst frequency | ω | 0.43 (0.33-0.66) min⁻¹ |
| Average RNAP2 arrival rate | τ = ω ⋅ β | 6.622 (5.74-27.43) min⁻¹ |
| Average time RNAP2 spends in the cluster | μcluster = 1/(kesc + kβ) | 0.692 (0.02-0.79) min |
| Average RNAP2 in the cluster | f = κesc/(κesc + kβ) | 4.624 (0.46-5.26) |
| Probability of RNAP2 in the cluster to escape | pRNA = f ⋅ β | 0.4615 (0.10-0.56) |
| Average mRNA burst size | | |
| Average mRNA production rate | | |
| Average actively transcribing RNAP2 | | |
| Average total RNAP2 at the transcription site | | |
| Average time for mRNA completion | | |
Discussion

In this study, we measured the dynamics of the RNAP2 CTD transcription cycle at the single-gene level in living cells. By combining complementary antibody-based imaging probes with multi-color single-molecule microscopy and computational modeling, we were able to detect organization in both the temporal ordering and spatial distribution of endogenous RNAP2 phosphorylation along a single HIV-1 reporter gene.

We find that a large number of RNAP2 at the HIV-1 transcription site are clustered around the promoter in a region that is spatially distinct from elongating RNAP2 and mRNA synthesis (as depicted in Fig. 5). This spatial organization supports the notion of dynamic RNAP2 clusters that form transcriptional hubs or factories that contain high concentrations of the transcription machinery. In steady-state, we estimate there is an average of ~20 RNAP2 at the HIV-1 gene. This total number of
Fig. 4 Intensity fluctuations of CTD-RNAP2, Ser5ph-RNAP2, and mRNA in the presence of transcription inhibitors. a Sample cell before the addition of Triptolide (TPL). The transcription site (TS) is shown in the dotted box, and the inset shows a zoom-in (n = 10/4). b The TS from (a) at all times before and after the addition of TPL. c Normalized average T5 intensity over time of all the quantified cells for CTD-RNAP2 (red circles), Ser5ph-RNAP2 (green squares), and mRNA (blue diamonds) before and after application of TPL (vertical dashed line). The inset shows the signals in a representative cell. Data are presented as mean values ± S.E.M. Significance was tested using a two-tailed Mann-Whitney U test with p ≤ 0.05 (*) and p ≤ 0.01 (**). d-f Normalized intensity signals after application of various transcription inhibitors, including d TPL (5 μM), e THZ1 (15 μM), and f Flavopiridol (Flav, 1 μM) for all the cells analyzed. Signals highlighted in black correspond to a sample single cell, the colored shadow and the full line in the middle of it correspond to the S.E.M. and the mean in each channel, respectively. The vertical gray dashed line in (e) highlights the time point at which a burst of RNAP2 is observed in the sample cell in the presence of THZ1. n = number of cells/number of independent experiments.

Fig. 5 Model depicting RNAP2 transcription dynamics in a single-copy gene. During extremely short (<<1 min) periods of the ON state, RNAP2 is recruited in bursts (~15 RNAP2) to the HIV-1 reporter gene, creating transient (~1 min) clusters of CTD-RNAP2 and Ser5ph-RNAP2 at the gene promoter, and initiating transcription in RNAP2 convoys (~7 RNAP2/convoy). The middle of the gene remains mostly empty due to rapid transcription, while a large number of RNAP2 (~15) concentrate at the end of the gene during processing (~4–5 min). In OFF periods (~2.3 min), RNAP2 convoys that escaped the promoter during the ON state quickly elongate and complete transcription. The gene rapidly transitions back to the OFF state when ON (denoted by arrows). ChIP assays enrich for genes with lots of RNAP2, which will bias the assay towards genes with RNAP2 clusters near the promoter.

RNAP2 is in between recent estimates of ~80 RNAP2 clustered at the constitutively expressed beta-actin locus, ~17 RNAP2 at an exogenous mini-gene, and ~7.5 RNAP2 at the Pou5f1 locus. Of the ~20 RNAP2 at our HIV-1 reporter gene, we estimate on average ~5 are at or near the promoter, awaiting initiation or promoter escape. During frequent bursts, however, this number can dramatically increase to as high as 90 RNAP2, with most either coming in with Serine 5 phosphorylation or rapidly acquiring Serine 5 phosphorylation within seconds. Given the limited amount of space at the promoter, it is hard to imagine all of these RNAP2 are promoter bound. Instead, we believe many are unbound and collectively this fraction helps form the transcription cluster, which remains spatially distinct from mRNA synthesis.

A major unresolved question is how RNAP2 is retained in clusters. One possibility is that RNAP2 is trapped by repeated interactions with other transcription machinery in the region. Alternatively, clusters could represent phosphorylation-dependent condensates. As others have recently shown, phase separation can be driven by phosphorylation of the unstructured RNAP2 CTD' and by the histidine-rich tail of P-TEFb. Since Tat directly interacts with P-TEFb, it could enhance RNAP2 recruitment and clustering at the HIV-1 reporter gene.

One possible advantage of the cluster is it retains recently aborted RNAP2 near the transcription start site so they can rapidly re-initiate. This follows from our rapid imaging experiments, which indicate initiation is very rapid (~3–6 s; Supplementary Fig. 5) compared to promoter escape (fitted 1/kesc ~1.5 min). The distinct timescales imply two hypotheses: First, most promoter escape attempts fail. This is consistent with earlier measurements based on FRAP that demonstrated successful promoter escape is a rare event. Second, a large fraction of RNAP2 in the cluster are inactive at any given time. Such a large fraction of inactive RNAP2 could arise from recently aborted molecules that retain their Ser5ph. Evidence for the retention of Ser5ph on RNAP2 after transcription abortion was seen in an earlier study, where Ser5ph-RNAP2 was detected in the soluble fraction of cells after transcription was globally inhibited via flavopiridol. The retention of RNAP2 also helps explain our model prediction that nearly half of the RNAP2 in the cluster (kesc/(kesc + kab) ~46%) eventually does escape the promoter and produce a full-length transcript. Thus, local recycling of transcription machinery within clusters may play a role in HIV-1 biogenesis, where Tat expression provides a positive feedback loop to amplify transcription and facilitate the rapid production of viral proteins in host cells.

While the overall efficiency of transcription is relatively high at the HIV-1 reporter gene compared to other genes studied, the various kinetic rates we quantified are fairly consistent with earlier work. In particular, we found RNAP2 takes around five
minutes to complete transcription after promoter escape (1/k_e in Fig. 3). This places an upper bound on the RNAP2 elongation and processing time. If we constrain the elongation rate to be 4.1 kb/min20 (~1 min for the full gene), then we can assign the remaining time (~4 min) to RNA processing at the 3’ ends. Under these conditions, the model predicts a build-up in RNAP2 at the 3’ end of the gene because processing takes longer than elongation. This build-up is consistent with our ChIP data in Fig. 1b. The estimated 4 min processing time is also consistent with an earlier estimate at this HIV-1 locus20, although such relatively long processing may not be representative of other genes. Similarly, the RNAP2 initiation and promoter escape rates we quantified are consistent with earlier reports, taking between a minute and a few minutes27,29. Finally, we also detected bursts in transcription that result in convoys of RNAP2, as previously reported20, and consistent with widespread bursting observed across the genome38,52.

The ability to image by fluorescence microscopy endogenous RNAP2 phosphorylation dynamics at single-copy genes now makes it possible to estimate the RNAP2 distributions predicted by ChIP. ChIP studies of the RNAP2 CTD transcription cycle typically display heterogeneous distributions of RNAP2 that have distinct peaks of Ser5ph-RNAP2 near the promoter and Ser2ph-RNAP2 at the ends of genes13,11. However, based on ChIP alone, it is not clear if peaks represent the distribution of RNAP2 along single genes or instead represent a population of genes. For example, it could be that half of the genes have Ser5ph-RNAP2 paused at the beginning of the gene, while the other half have Ser2ph-RNAP2 being processed near the end of the gene. In this extreme example, no single gene would have RNAP2 at both ends. According to our best-fit model, the situation for HIV-1 is not this extreme, but the distribution of RNAP2 does depend sensitively on the timing of bursts. For example, early in a burst RNAP2 occupancy is heavily front-loaded, with all or nearly all RNAP2 at or around the promoter in a Serine 5 phosphorylated conformation. Since RNAP2 ChIP by design is biased towards genes with high levels of RNAP2 at the time of assay, genes that have recently burst are likely to be overrepresented in the data (Supplementary Fig. 8b, c). As our model demonstrates, soon after a burst, genes tend to have far more RNAP2 clustered around the promoter than the average gene (which has just five) (Fig. 5). According to this interpretation, the large Ser5ph-RNAP2 ChIP peak we observe near the promoter could arise from rapid and repeated promoter–proximal initiation and/or pausing. Given the nature of ChIP, it is also possible the peak arises from RNAP2 within clusters that are non-specifically cross-linked during the fixation step. However, this latter possibility seems unlikely as promoter–proximal peaks are also observed using techniques that detect and sequence nascent mRNAs, such as GRO-seq, PRO-seq, and mNET-seq43. In the future, it will be interesting to see to what extent dynamic clustering observed in living cells correlates with promoter–proximal RNAP2 peaks observed across the genome in populations of fixed cells34.

Aside from HIV–1, our technology can now be used to examine RNAP2 phosphorylation dynamics at other single-copy genes. Given the high correlation between MS2 (mRNA) and RNAP2 (Fabs), in the future MS2 may not even be required. For example, by combining Fab and CARGO35, RNAP2 phosphorylation dynamics at an endogenous gene could be visualized without extensive genome editing. Alternatively, Fab could be combined with other labeling technologies such as lacO/lacF56,57, ROLEX36, ANCHOR39, or post-fixation via DNA FISH60 or CasFISH61. Beyond RNAP2, post-translational modifications to other proteins involved in transcription could also be studied in this way, including histones23,62. However, a few important caveats of Fab- or intrabody-based imaging should be kept in mind: First, if Fab bind their targets with too low affinity, then there will be a large unbound fraction that will decrease signal-to-noise. For the CTD-RNAP2 and Ser5ph–RNAP2 Fab, the bound fraction was determined to be greater than 80%67. Second, if Fabs take too long to bind their targets, then very rapid processes can be entirely missed or their timescales will appear erroneously slow. According to FRAP, the vast majority of CTD-RNAP2 and Ser5ph–RNAP2 Fabs used in this study bind and rebinding their targets in well under 10 s67, meaning processes on the seconds time scale can be discerned, but anything shorter may be missed. Third, if Fabs are too numerous in a cell, they may compete with one another for binding, and Fab targets could become saturated, both of which could interfere with the underlying biology. We introduce 1–3 × 10^6 Fab per cell52, far less than the ~1.5 × 10^6 RNAP2 heptad repeats57,62. We therefore do not expect Fabs to compete or interfere. Together these three caveats place considerable constraints on the experiments, but they are not prohibitive. With the continued development of Fab33, scFv44, and nanobodies44 for live-cell imaging, finding a suitable intrabody has become significantly easier. We therefore anticipate our technology will become a valuable tool to study transcription dynamics at the single-gene level.

**Methods**

**Cell culture**. Transcription dynamics experiments were performed in HeLa Flp-in H9 cells (H-128). The H-128 cell line generation was described previously59. Briefly, H-128 cells harbor an HIV-1 reporter gene tagged with an MS2X128 cassette, controlled by Tat expression. The HIV-1 reporter comprises the 5’ and 3’ long terminal repeats containing the viral promoter, polya’s sites, as well as HIV-1 splice donor (SD1), splice acceptor (SA7), and Rev-responsive element. H-128 cells also stably express MCP tagged with GFP (MCP-GFP), which binds to MS2 repeats when they are transcribed into mRNA. Cells were maintained in a humidified incubator at 37 °C with 5% CO2 in Dulbecco’s modified Eagle medium (DMEM, Thermo Fisher Scientific, 11960-044) supplemented with 10% fetal bovine serum (FBS, Atlas Biologicals), 10 μg/mL penicillin/streptomycin (P/S, Invitrogen), 1 mM l-glutamine (l-glut, Invitrogen) and either 400 μg/mL Neomycin (Invitrogen) or 150 μg/mL Hygromycin (Biotechnext).

**Chromatin immunoprecipitation and quantitative-polymerase chain reaction (ChIP-qPCR)**. ChIP was performed as described previously60 with minor modifications. Briefly, H-128 cells grown in a 10 cm dish were fixed with 1% PFA in PBS at room temperature at 5 min, neutralized in 5 mM EDTA, sheared 200 mM glycine for 5 min, and washed with PBS and NP-40 buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl and 0.5% NP-40). Fixed cells were lysed with 360 μl sodium dodecyl sulfate (SDS) dissolution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA and 1% SDS) and diluted with 1440 μl ChIP dilution buffer (50 mM Tris-HCl, pH 8.0, 167 mM NaCl, 1.1% Triton 100x and 0.11% sodium deoxycholate), supplemented with a protease inhibitor cocktail. After shearing chromatin a Bioruptor UCD-200 (Diagenode) at sonications of 40 sec with 50 sec intervals, eight times at high level, the median size of fragmented DNA was 200 base pairs with a range of 50–500 base pairs. The supernatant, cleared by centrifugation at 20,000 xg for 10 min at 4 °C, was diluted with 5.4 μl ChIP dilution buffer and then incubated with 40 μg sheep anti-mouse IgG magnetic beads pre-incubated with 1 μg mouse anti-CTD-RNAP2 (MABI 0601), anti-Ser5ph–RNAP2 (MABI 0603), and anti-Ser2ph–RNAP2 (MABI 0602) monoclonal antibodies (Cosmo Bio USA) at 4 °C overnight with rotation. The immune complexes were washed with low-salt RIPA buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton 100x and 0.1% sodium deoxycholate), high-salt RIPA buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 500 mM NaCl, 0.1% SDS, 1% Triton 100x and 0.1% sodium deoxycholate) and then washed twice with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). DNA was eluted with ChIP elution buffer (10 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5 mM EDTA and 0.5% SDS). After incubation at 65 °C overnight to reverse the cross-links, DNA was purified by RNase A and proteinase K treatments and recovered using a DNA purification kit (Qiagen). For ChIP-qPCR, the immunoprecipitated DNA and total DNA were quantified by Power SYBR Green PCR Master Mix in an Mx3000P Real-Time qPCR System (Agilent Technologies). The primers used for qPCR are listed in Supplementary Table 1.

**Antigen-binding fragment (Fab) preparation and fluorescence conjugation**. Fab preparation was performed using the same monoclonal antibodies used in ChIP experiments and the Pierce Mouse IgG1 Fab and F(ab’2) Preparation Kit (Thermo Scientific).
Scientific), as described before29. In brief, fixin resin was equilibrated with 25 mM 
arginate (in HCl, pH 5.6) to digest the antibodies (CTD-RNAP2 or Ser5ph- 
RNAP2) into Fab. The IgG concentration used was 4 mg, and the digestion re-
action was incubated for 5 h. Fab and Fc regions were separated using a Nab Protein
A column (Thermo Scientific). Fab’s were concentrated up to ~1 mg/mL using an
Amonica Ultra 0.5 filter (10 k cut-off, Millipore) and conjugated with CF640 or Cy3
(Invitrogen) dyes. For labeling Fab, 100 μg of purified Fab and 10 μL of 1M 
NaHCO3 were mixed to a final volume of 100 μL, then 2 μL of CF640 or 2.66 μL of 
Cy3 was added, and the mixture was incubated at RT for 2 h in a rotator protected
from the light. The labeled Fab sample was passed through a PD-mini G-25
desalting column (GE Health care), previously equilibrated with PBS, to remove
unconjugated Fab, and then the dye-conjugated Fab was concentrated up to ~1
mg/mL with an Amicon Ultra filter 0.5 (10 k cut-off). The degree of labeling (DOL)
calculated using Eq. (1), where εFab and εCy3 are the extinction coefficients of
IgG at 280 nm and the dye (provided by the manufacturer), AFab and ACy3 are the
absorbances determined at 280 and 650 or 550 nm, and CF is the correction factor
for the dye at 280 nm (provided by the manufacturer). In this study, only Fab’s with a
DOL between 0.75 and 1 were used for live-imaging experiments.

\[
\text{DOL} = \frac{\epsilon_{\text{Fab}}}{\epsilon_{\text{Cy3}}} \left( \frac{A_{\text{Fab}}}{A_{\text{Cy3}}} - CF \right)
\]  

Loading fluorescent Fabs into living cells. Cells were cultured in glass-bottom
dishes (35 mm, 14 mm glass, Mat-Tek). The next day dye-conjugated Fabs were
loaded into the cells through bead-loading24,25, as follows: First, the fluores-
cent dye Cy3 (Invitrogen), CF640 (Invitrogen), and HaloTag TMR Ligand (5 mM) (Pro-
teinTech) were dissolved in DMSO (Sigma-Aldrich) and stored at
–20°C until use. RNAP2 inhibitors were added to the DMEM medium to reach the desired final
concentration in the cells.

Chemicals. The transcription inhibitors, Triptolide (TPL, Sigma Aldrich), Flavo-
piridol (Flav, Selleck Chemicals), THZ2, (Selleck Chemicals), fluorescence dyes,
Cy3 (Invitrogen), CF640 (Invitrogen), and HaloTag TMR Ligand (5 mM) (Pro-
tech) were mixed with PBS up to 4 μL in the cell culture hood. Second, the medium
was removed completely from the dish and stored, and the Fab mixture was added
to the center of the dish. Third, glass beads (106 μm, Sigma-Aldrich, G-4649) were
immediately sprinkled on top before cells dried up and the dish was tapped ~10
times against the bench. This tapping causes the beads to roll over cells and induce small
bubbles that will help the dye to diffuse into the cells. Fourth, the stored medium was
quickly added back to the cells, again to prevent cells from drying out. Cells were
then placed in the incubator to recover for 1–2 h. Post-recovery, the glass beads
were gently washed out with phenol red-free DMEM (DMEM+, Thermo Fisher
Scientific, 31053-028), and the cells were stored in DMEM medium (DMEM+ supple-
mented with 10% FBS, 10 μM P/S, and 1 mM L-glut) for live-imaging experiments.

Microscopy. A custom-built widefield fluorescence microscope with highly
inclined illumination was used in all experiments13,30. The microscope has three excitation
beams: 488, 561, and 637 nm solid-state lasers (Votram) that are coupled and focused
onto a back focal plane of the objective (Olympus, N.A 1.46 oil immersion
objective, Olympus). The emission signals were split by an imaging grade, ultra-flat
dichroic mirror (T6601pxr, Chroma) and detected with two aligned EM-CCD
cameras (EM-CCD, Hamamatsu). The laser power at the back focal plane was set to 21.4, 60.5, and 21.74
μW for 488, 561, and 637 nm, respectively. The wavelength of the excitation lasers,
CTD-RNAP2 in red (CF640), Ser5ph-RNAP2 in purple color distribution based upon their average Euclidean
displacement for each track longer than 5 frames. Tracks were plotted with a blue–purple color distribution based upon their average Euclidean
displacement. The transcription site was identified using TrackMate and plotted in red.

Live-cell imaging of transcription at the HIV-1 reporter gene. To cover the
total volume of the cells, all movies were taken using a 10× objective (NA 0.4) with
0.5 μm thick image. The x-y plane was moved only after all three colors were imaged in each plane.
This was followed in the x-y plane by 0.08 μm for each frame. The image of all colors for each frame
was inverted to visualize the color distribution based upon their average Euclidean

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displacement. The transcription site was identified using TrackMate and plotted in red.

Immunoﬂuorescence. Cells grown on glass-bottom dishes (35 mm, 14 mm glass,
uncoated, Mat-Tek Corporation) were ﬁxed with 4% Paraformaldehyde (Electron
Microscope Sciences) in 1 M HEPES (Sigma-Aldrich) with or without 10% 
Trition-100% (Fishier Scientiﬁc) (PH 7.4) for 10 min at RT, and washed with PBS (3×). 
Permeabilization (1% Triton 100× in PBS) and blocking (100% blocking One-P, 
Nacalai-US) were performed individually, for 20 min at RT, gently rocking, and
with PBS (3×) after each step. The cells were incubated for 2 h at RT with
with PBS (3×) after each step. The cells were incubated for 2 h at RT with
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Quantifying signal intensities at the transcription site from live-cell imaging movies. Images were pre-processed using either Fiji or custom-written batch processing Mathematica code (Wolfram Research 11.1.1) to create 2D maximum intensity projections from 3D movies. Using Mathematica code, the 3D images were corrected for photobleaching and laser fluctuations, x-stack by x-stack, by dividing a movie by the mean intensity of the whole cell or the background channel. The offset between the two cameras was registered using a built-in Mathematica routine FindGeometricTransform, which finds a transformation that aligned the best-fitted positions of 100 nm diameter Tetraspeck beads evenly distributed across the image field of view. 2D maximum projections and 3D image sequences from the images corrected for bleaching and laser fluctuations were then analyzed with a custom-written code in Mathematica to detect and track the transcription site. Briefly, thresholds were selected in each channel to visualize spots at the transcription site and a bandpass filter was used to highlight just the transcription site in the mRNA channel. The resulting binary image was binarized and used to create two masks for each time point: one marking the transcription site (TS mask: a mask semi-manually thresholded to cover just the transcription site within the image) and one marking the BG (mask: a ring of width one pixel that surrounds the transcription site and is separated from the site by two pixels). The built-in Mathematica routine ComponentMeasurements-IntensityCentroid was used to find the coordinates of the transcription site in XY through time. The Z coordinate was determined by selecting the z-stack in which the particle in the XY coordinate had its maximum brightness (“best z”). If the transcription site disappeared (due to transcription turning off or inhibition), the Z replaced by the Z coordinate of the last visible position. From the XYZ coordinates at each time point, a 2D maximum projection was created considering the “best z” at each time point. From this, the pixel intensity values were recorded for each TS and BG mask, representing the mean intensity values over time at the transcription site and the BG, respectively. The raw and normalized intensity vectors were calculated per channel and a moving average of three-time points was used to display the intensity RawIntachi as a function of time, as shown in Eq. (2):

\[
\text{RawIntachi} = (I_{\text{ach}}(t) - I_{\text{ach}}(\tau))
\]

where, \(I_{\text{ach}}\) is the intensity measured in the TS mask in each channel (Ch), \(I_{\text{BG}}\) is the intensity measured in the BG mask, and \(\tau\) represents a three-point time moving average. The normalized intensity (as in Eq. 2c) was calculated by dividing RawIntachi by the average 95% intensity from all transcription sites. Occasionally, normalized intensities for CTD-RNAP2 and Ser5ph-RNAP2 dip below zero. This can be caused either by RNAP2 signals being temporally depleted at the transcription site vs. off-target at a random site \(p_1\). Significance was calculated using the Mann–Whitney U test.

Analysis of minima signals in Fig. 2d. The local minima in the mRNA signal of each cell were detected using the "localmin" function in MATLAB. The cells that exhibited minima below a threshold (normalized intensity ≤ 0.02 arb.units) were selected by the algorithm. Then seven-time points before and after the mRNA valley were considered, including the minimum, in each channel. All the traces in each channel were averaged and fitted with a Gaussian using a 95% confidence interval to determine the minima and maximum steady state of the average trace in each channel.

To confirm that the minima were true and not an artifact of our analysis, the analysis was repeated at hundreds of random time points. Significance was calculated using the Mann–Whitney U test. The \(p\) values for the magnitude of the minima the three-time delay times were calculated by comparing the magnitude of the minima to the control and the time lag to minute zero in each signal, respectively.

Analysis of transcription site spatial organization in Figs. 2e-h and Supplementary Fig. 3h. Moving average (30 time points) movies were generated to accurately determine the mean XY position of the transcription site in each channel. As described in Quantifying signal intensities at the transcription site from live-cell imaging movies, the raw intensity channels were semi-manually thresholded to detect and track the transcription site. ComponentMeasurements-IntensityCentroid was used. Once the XY positions for each signal were obtained, the Euclidean distance between each pair over time was calculated, from which distributions were calculated. Significance between signals was calculated using the Mann–Whitney U test.

Auto- and cross-correlation analysis. The auto- and cross-correlation functions were calculated for each time trace obtained from the longer movies (like in Fig. 2c, but without performing a 3-time point moving average), as previously described.\(^{14,15}\) The covariance function is defined as:

\[
G(r) = \langle \delta t \delta b(t + r) \rangle
\]

where \(\langle \cdot \rangle\) indicates the temporal mean, and \(\delta t\) denotes the deviation about the mean, i.e., \(\delta t(t) = (t(t) - \langle t(t) \rangle)\). Signals \(a(t)\) and \(b(t)\) can be the same signal or two different signals. In the first case, \(a = b\) and \(G(r)\) represent the auto-covariance, which is symmetric about \(r = 0\); In the second case, \(G(r)\) represents the cross-covariance and may be asymmetric. To calculate the cross-correlation between the CTD-RNAP2 and Ser5ph-RNAP2 signals in the fast imaging experiments in Supplementary Fig. 5, the intensities of tracked transcription sites through time were quantified as described above, with a couple of minor modifications: First, because imaging was in a single plane, the rate of photobleaching in the plane was not captured by the rate of photobleaching in the cell. For this reason, each signal exponentially decayed. This was corrected by dividing out a single-exponential fit to each curve. Second, we did not perform any moving average on the signals to maintain the highest possible temporal resolution.

For fitting and data analysis, the normalized covariances, \(G(r)/G(0)\), were used for all signals, where \(G(0)\) denotes the zero-lag auto- or cross-covariance averaged over all time points and all biological replicas. To quantify and remove shot noise from the zero-lag auto-covariances, \(G(0)\) was estimated for each biological replica assuming a linear interpolation from the three shortest non-zero lag times (1, 2, 3 min) prior to averaging over all replicas. The standard error of the mean normalized covariance functions, denoted \(\text{SEM}_G(r)\), was computed as the standard deviation of \(G(r)/G(0)\) divided by \(\sqrt{N}\).

A quantitative model for transcription. The derivation of the bursting model for RNAP2 recruitment and nascent transcription simple model begins with the specification of three variables: \(x(t)\) describes the promoter state, \(x(t)\) describes the number of RNAP2 in the cluster, and \(x(t)\) describes the number of RNAP2 engaged in active transcription. Six reactions can occur: (1) a promoter can become temporarily active with propensity equal to the burst frequency, \(\omega - k_{\text{act}}\) the active promoter can deactivate at a rate \(k_{\text{off}}\), (3) the active promoter can recruit and phosphorylate RNAP2 at Ser5ph-RNAP2 dip below zero. This can be caused either by RNAP2 signals being temporally depleted at the transcription site vs. off-target at a random site \(p_1\). Significance was calculated using the Mann–Whitney U test.

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To convert these above expressions, which are in terms of $x_1 - x_2$, into quantities reflecting the total RNA2P at the transcription site ($y_1 = x_2 + x_3$) and number of transcribing RNA2P ($y_2 = x_3$), we define a simple linear transformation

$$
y = \begin{bmatrix}
0 & 1 & 1 \\
0 & 1 & 1 
\end{bmatrix} \mathbf{x},
$$

(11)

Under this transformation, $E[y|x] = E_x[y|x] = E_x[y|x] = E[x]T$. We note that this version of the model does not distinguish between RNA2P and Ser5ph-RNA2P. These two distinct forms as well as other configurations are easily incorporated by extending x to include a fourth or more states. In such cases, each new state adds two reaction stoichiometry vectors to Eq. (4), two reaction terms to Eq. (5), and one additional column to the output matrix $\mathbf{c}$ in Eq. (11), but the rest of the analysis remains unchanged.

Using this model formulation, it is straightforward to solve for the steady-state moments (Eqs. (8) and (9)) and the auto- and cross-correlations (Eq. (10)) for any combination of parameters. However, upon fitting this model to the data, we observed that estimates for $k_{cat}$ tended to very large values ($k_{cat} \gg \omega$) and with substantial estimation uncertainty. Under these excessively large rates for $k_{cat}$, each “on” period is extremely short-lived and attracts a geometric number of RNA2P with mean $\beta$ (this model reduction is equivalent to the strategy in models that use Gaussian approximation such that

$$
\sigma^2 = \text{err}^2, 
$$

where $\sigma$ is the sample mean levels of mRNA from the data, $\mu_0$ is the mean number of mRNA predicted by the model, and $\text{SEM}_0 = 0.93$ is the standard error of the mean level of mRNA from the data. Similarly, the log-likelihood of the measured variance, $\sigma_0^2$, given the model was estimated as

$$
\log L_0(\theta) = C_0 - \frac{1}{2} \sum_{i=1}^{\text{lag}} \left( \frac{\text{SEM}_0^2}{\sigma_0^2} - 1 \right). 
$$

where $\mu_0$ is the sample mean levels of mRNA from the data, $\mu_0(\theta)$ is the mean number of mRNA predicted by the model, and $\text{SEM}_0$ was estimated at 1000 sec (this model reduction is equivalent to the strategy in models that use Gaussian approximation). The mRNA retention model has one additional parameter (i.e., $k_{cat}$ was replaced with $k_{ret}$). The model selection procedure toward simpler models—larger choices of $n$ would result in much stronger rejection of the more complex models.

**Transcription inhibition experiments.** For the transcription inhibition experiments in Fig. 4, cells were imaged every 1 min for 5-time points before applying the inhibitor ($t = 0$). TPL (5 μM), THZ1 (15 μM), or Flav (1 μM). Cells were then imaged every 1 min for 30 min total after addition of TPL or Flav, and for 55 min total after addition of THZ1. Here, laser power at the objective’s back focal plane was set to 21.4, 60.5, and 21.74 μW for 488, 561, and 637 nm, respectively, and the exposure time was 53.64 msec.

To quantify time delays in the TPL-runoff assay, TPL signals were further analyzed as follows: (1) To account for cell variability and experimental conditions, the decays curves from each cell were aligned. This was achieved by subtracting the time at which each cell reached half of the decay after TPL addition. This time was obtained by an inverse hyperbolic tangent fit applied to each channel in every cell (Fig. 4c); (2) after the alignment, all the traces in each channel were averaged together, and the standard error of the mean (S.E.M.) was calculated. Finally, to determine the time delays between CDT-RNA2P, Ser5ph-RNA2P, and mRNA, an inverse tanh fit was applied and weighted with respect to the variance of each signal.

**Software.** All images were acquired with Micro-Manager software (1.4.22). Image pre-processing was made using ImageJ (2.0.0). All figures were deposited to Figshare [https://doi.org/10.5281/zenodo.4631141]. The Mathematica codes employed for the image processing in this paper are available from the authors upon request.

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**Competing interests**

The authors declare no competing interests.

**Additional information**

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