SUPPLEMENTARY ONLINE MATERIAL FOR DUNDR ET AL.

Materials and Methods

Constructs
A full-length 1953-bp clone of human TIF-IA/Rrn3 was inserted as an XhoI-
BamHI fragment into the pEGFP-C1 vector (Clontech). A full-length 1305 bp
clone of mouse PAF53 was inserted a KpnI-BamHI fragment into pEGFP-C1. A
full-length 1362 bp clone of mouse TAF48 was inserted as a SacI-KpnI fragment
into pEGFP-N3. A full-length 402 bp clone of human RPA16 was inserted as an
EcoRI-EcoRI fragment into pEGFP-N3. A full-length 1041 bp clone of human
RPA40 was inserted as an EcoRI-EcoRI fragment into pEGFP-N3. A full-length
993 bp clone of human RPA43 was inserted as an EcoRI-EcoRI fragment into
pEGFP-N3. A full-length 4125 bp clone of mouse RPA194 was inserted as a PseI-
ApaI fragment into modified pEGFP-C2. Human GFP-UBF1 and GFP-UBF2
subcloned into pEGFP-C1 was a generous gift from Drs. D. Ploton and M.F.
O'Donohue (Reims, France). Human GFP-UBF1-N-box3 mutant was a generous
gift from Dr. Sui Huang (Chicago, IL).

Cell Culture and Transfection
Monkey CMT3 cells were grown in DMEM (Invitrogen) supplemented with 10%
FCS (Hybaid), 1% glutamine, and penicillin and streptomycin at 37°C in 5% CO2.
The cells were electroporated with a BTX electroporator ECM 830 using 5 µg
plasmid DNA and 15 µg sheared salmon sperm carrier DNA in a 2 mm gap
cuvette. The electroporation setting was 150 V, 1 ms pulse, 4 pulses and 0.5 sec
between intervals. After electroporation the cells were plated in Nalgene Lab
Tek II chambers and after ~ 6 hours the medium was changed to DMEM with 25
mM Hepes without phenol red.
In Situ Hybridization

The 5'ETS core region of human pre-rRNA, which corresponded to a SacI-KpnI fragment of human pre-rRNA (nt +934/+1,444) in pBluescript SK(-) was linearized with XhoI and the antisense hybridization probe (+1,270/+1,444) was produced by in vitro transcription with biotin-16-UTP using T7 RNA polymerase. In situ hybridization was carried out as described (S1). Samples were observed on a Zeiss LSM 510 laser scanning microscope using a 100x, 1.3 N.A. oil planapochromat objective.

Visualization of transcription sites

Transcription sites were labeled as described by Elbi et al. (S2). Br-UTP incorporation was for 8 min. Cells were mounted in Mowiol (Calbiochem) and observed on a Zeiss LSM 510 laser scanning microscope using a 100x, 1.3 N.A. oil objective.

Pull down of RNA pol I-GFP fusion proteins

Mouse 3T3 F5 cells stable expressing Rrn3-FLAG were transfected with pol I-GFP fusion proteins using Lipofectamine2000 (Gibco/BRL) for 36 h. Whole cell lysate were prepared using lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease inhibitors (Roche). 180 µl aliquots were incubated at 4°C with 25µl anti-FLAG agarose beads and mixed for 2 hr at 4°C. The beads were washed with lysis buffer, then washed three times with FLAG wash buffer (50 mM Tris HCl pH 8.0, 238 mM NaCl, 27 mM KCl, , 20% glycerol), separated by 7.5% SDS-PAGE, transferred to Immobilon P and probed with a polyclonal anti-GFP antibody (Clontech). The antigens were visualized by the Enhanced Chemiluminescent (ECL) method (Amersham).
FRAP and iFRAP

Fluorescence recovery after photobleaching (FRAP) experiments were performed on Zeiss 510 confocal microscope with a 100x/1.3 N.A. planapochromat oil objective. GFP was excited with the 488 nm line of Ar laser and GFP emission was monitored above 505 nm. Cells were maintained at 37°C with a Nevtek ASI 400 Air Stream incubator (Nevtek). In transfected cells a circular spot of ~ 1 µm in diameter inside of the nucleolus was bleached for ~ 50ms using the 488 nm laser line at 100% laser power. Cells were monitored in 1s intervals for GFP-UBF1, GFP-UBF2, TAF48-GFP and 2s intervals for all other GFP fusion proteins. For quantitation, the total fluorescent intensities of a region of interest in the bleached area and in the total nuclear area were monitored using Zeiss software. Background fluorescence was measured in a random field outside of the cells. The relative fluorescence intensity double normalized to the pre-bleach value was calculated as described (S3). In inverse FRAP (iFRAP) experiments the whole nuclear area of a transfected cell was bleached with the exception of a small region typically containing one to three nucleolar fibrillar centers. For bleaching the 488 nm laser line at 100% laser power was used and a typical bleach time was ~ 700ms. Cells were monitored in 2s intervals. For each time point the relative loss of total fluorescent intensity in the unbleached region of interest was calculated as:

\[ I_{rel} = \frac{I(t)}{(I_0)^r(T(t))} \]

where \( I(t) \) is the average intensity of the unbleached region of interest at time point \( t \), \( I_0 \) is the average pre-bleach intensity of the region of interest and \( T(t) \) is the average total nuclear fluorescence intensity of a neighboring cell in the same field of view. In all FRAP and iFRAP experiments more than 20 cells from at least two independent experiments were quantitated.
Formulation of the kinetic model

Polymerase I transcription was assumed to be in steady state and the various GFP fusions of polymerase subunits and initiation factors were assumed to obey the tracer assumption. This means that the GFP tagged proteins were assumed to participate in cellular events as their endogenous counterparts. This assumption is supported by experimental evidence described in the Results section. As a consequence of the tracer assumption all processes can be treated as linear first order reactions because the steady state values of the untagged partners in second order reactions are constant.

The following states (also referred to as compartments or state variables) were included in the model described in Figure 4a of the main text: 1) entry into the FC from the nucleoplasm, 2) release from the FC back to the nucleoplasm, 3) recruitment to the promoter from the FC, 4) release from the promoter back to the FC, 5) initiation of transcription, 6) a cascade of processes representing elongation connecting a serial arrangement of states representing polymerases at various positions along the rDNA gene, 7) termination with release of the Pol I subunits back to the nucleoplasmic pool, 8) entry of cytoplasmic proteins into the nucleus, 9) exit of proteins from nucleoplasm to cytoplasm.

The differential equations describing this model are included here (in standard FORTRAN-like notation) in their entirety to facilitate replication and extension of this work. The kinetic model used in fitting the experimental data is identical to the one shown in Figure 4a except that it was replicated to represent both molecules in the bleached fraction of the nucleus (FkB = Fraction of bound molecules Bleached, FfB = Fraction of free molecules Bleached) and in the unbleached fraction. Symbols beginning with B represent the bleached states. The nucleoplasmic compartment was assumed well-mixed on the time scale of
these experiments (500 seconds). The well-mixed behavior of the nucleoplasmic compartment has been confirmed by FRAP and FLIP experiments (T. Misteli, unpublished data). Symbols beginning with k are first order rate constants for the corresponding process; each has units of sec$^{-1}$. With no loss of generality, all of the constant steady state values of binding partners and other reactants, as well as all volumes of distribution were included in these rate constants. Notice that all rate constants are exactly the same in the bleached and unbleached portions of the nucleus; replicating the model to account for bleached and unbleached molecules adds no new unknown parameters. Of these constants, only one, kbleach, is not shown in the model diagram of Figure 4a. This constant is the bleach constant for the laser photobleaching protocols. It removes fluorescent material from all “B” states with exponential kinetics during any interval when the bleaching laser is on (typically ~ 50ms for FRAP, 700ms for iFRAP), as has been demonstrated experimentally (S3). We arbitrarily introduced ten elongation states, elong1 through elong10, in the model to approximate the distribution of transcribing polymerases along the length of the rDNA. Cellular place names for all proteins are npl = nucleoplasm, cyto = cytoplasm, FC = fibrillar center, Pol1promoter = promoter, elong = elongation.

The differential equations describing this system are:

$$\frac{d}{dt}(BFC) = kf*Bb*npl - kr*BFC - kon*BFC + koff*BPol1promoter - kbleach*BFC$$

$$\frac{d}{dt}(BPol1promoter) = kon*BFC - koff*BPol1promoter - kstart*BPol1promoter - kbleach*BPol1promoter$$

$$\frac{d}{dt}(Belong1) = kstart*BPol1promoter - kelong*Belong1 - kbleach*Belong1$$

$$\frac{d}{dt}(Belong2) = kelong*Belong1 - kelong*Belong2 - kbleach*Belong2$$

$$\frac{d}{dt}(Belong3) = kelong*Belong2 - kelong*Belong3 - kbleach*Belong3$$

$$\frac{d}{dt}(Belong4) = kelong*Belong3 - kelong*Belong4 - kbleach*Belong4$$
\[
\frac{d}{dt}(\text{Belong}5) = \text{kelong} \cdot \text{Belong}4 - \text{kelong} \cdot \text{Belong}5 - \text{kbleach} \cdot \text{Belong}5 \\
\frac{d}{dt}(\text{Belong}6) = \text{kelong} \cdot \text{Belong}5 - \text{kelong} \cdot \text{Belong}6 - \text{kbleach} \cdot \text{Belong}6 \\
\frac{d}{dt}(\text{Belong}7) = \text{kelong} \cdot \text{Belong}6 - \text{kelong} \cdot \text{Belong}7 - \text{kbleach} \cdot \text{Belong}7 \\
\frac{d}{dt}(\text{Belong}8) = \text{kelong} \cdot \text{Belong}7 - \text{kelong} \cdot \text{Belong}8 - \text{kbleach} \cdot \text{Belong}8 \\
\frac{d}{dt}(\text{Belong}9) = \text{kelong} \cdot \text{Belong}8 - \text{kelong} \cdot \text{Belong}9 - \text{kbleach} \cdot \text{Belong}9 \\
\frac{d}{dt}(\text{Belong}10) = \text{kelong} \cdot \text{Belong}9 - \text{kterm} \cdot \text{Belong}10 - \text{kbleach} \cdot \text{Belong}10 \\
\frac{d}{dt}(npl) = -\text{kf} \cdot npl + \text{kterm} \cdot \text{Belong}10 + \text{kterm} \cdot \text{elong}10 - \text{kbleach} \cdot FfB \cdot npl + \text{kr} \cdot FC + \text{kr} \cdot BFC - \text{knes} \cdot npl + \text{knls} \cdot \text{cyto} \\
\frac{d}{dt}(\text{cyto}) = \text{knes} \cdot npl - \text{knls} \cdot \text{cyto} \\
\frac{d}{dt}(\text{FC}) = \text{kf} \cdot (1 - \text{FbB}) \cdot npl - \text{kr} \cdot FC - \text{kon} \cdot FC + \text{koff} \cdot \text{Pol1promoter} \\
\frac{d}{dt}(\text{Pol1promoter}) = \text{kon} \cdot FC - \text{koff} \cdot \text{Pol1promoter} - \text{kstart} \cdot \text{Pol1promoter} \\
\frac{d}{dt}(\text{elong}1) = \text{kstart} \cdot \text{Pol1promoter} - \text{kelong} \cdot \text{elong}1 \\
\frac{d}{dt}(\text{elong}2) = \text{kelong} \cdot \text{elong}1 - \text{kelong} \cdot \text{elong}2 \\
\frac{d}{dt}(\text{elong}3) = \text{kelong} \cdot \text{elong}2 - \text{kelong} \cdot \text{elong}3 \\
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\frac{d}{dt}(\text{elong}9) = \text{kelong} \cdot \text{elong}8 - \text{kelong} \cdot \text{elong}9 \\
\frac{d}{dt}(\text{elong}10) = \text{kelong} \cdot \text{elong}9 - \text{kterm} \cdot \text{elong}10
\]
For all iFRAP experiments, the unbleached cytoplasmic protein was found to be a sufficient explanation of the late plateau in the experimental data. As it is experimentally impractical to bleach the large, albeit dilute, cytoplasmic pool, entry of these molecules produces an unavoidable background below which intranuclear events cannot be detected late in the experiment.

**Optimization and sensitivity analysis**

This model was coded in the Berkeley Madonna differential equation solver package ([www.berkeleymadonna.com](http://www.berkeleymadonna.com)) and could be quickly solved using standard fixed or variable step size numerical integrators. Algebraic equations were written to relate the model states to the observed experimental data and the model parameters were adjusted using the Levenberg-Marquardt optimization algorithm that is incorporated in Berkeley Madonna. Since Berkley Madonna lacks the tools to propagate noise in experimental measurements into estimates of variance for the model parameters, we optimized fits by approaching the least squares solution from several points in parameter space with the objective of avoiding local minima on the sum of squares hypersurface.

Thus while we can confidently assert that the model is quantitatively consistent with the experimental data, we cannot quantify the confidence in the numerical values of the rate constants. To address this, we performed several types of sensitivity analysis.

First we explored the sensitivity of the best-fit analysis to elongation time. The result, shown in Figure 4c of the main text, shows that if the model structure is correct then it is very unlikely that the correct elongation time is very different from our estimate of 140 – 150 seconds. Discrepancies in the fit are quite apparent at elongation times of 100 or 200 seconds, and elongation times of 10, 50, 250, or 300 seconds are completely inconsistent with the experimental data on
iFRAP of GFP-RPA194. A second, complementary and more formal approach to sensitivity is to calculate a sensitivity function for each of the parameters of interest. One common expression of sensitivity is obtained by comparing the best-fit curve to the one obtained when a parameter is incremented by $\Delta P$ where $P$ is the best-fit parameter value, and then calculating

$$S(t) = \frac{V(P + \Delta P, t) - V(P, t)}{\Delta}$$  \hspace{1cm} (1.1)$$

This sensitivity (Eq. 1.1), calculated for each of the model’s rate constants using $\Delta=0.001$, shows that the slow decline of the iFRAP is dominated by $k_{elong}$. Sensitivity to $k_{elong}$ increases at just the point of inflection for this slow decline, and then decreases again when the cytoplasmic plateau is reached. Sensitivities for all the remaining parameters, except $k_{nls}$, are monotonically decreasing over the course of 500 seconds and therefore affect the early shape of the rapid decline far more than the slow decline we are attributing to elongation. The nuclear import rate constant, $k_{nls}$, has an almost constant influence over the full 500 seconds and therefore cannot differentially affect this portion of the curve.

A similar approach was used to evaluate our conclusion that once a polymerase subunit is on the promoter, initiation probability ($P_{\text{initiation}}$) is only 5-10%. Variation of the initiation probability by calculating $k_{\text{start}}$ as

$$k_{\text{start}} = \frac{k_{\text{off}} P_{\text{initiation}}}{1 - P_{\text{initiation}}}$$  \hspace{1cm} (1.2)$$

demonstrated, as described in the main text, that best fits required $P_{\text{initiation}} < 0.1$. Initiation probabilities $> 0.2$ were entirely inconsistent with the experimental data, especially in the first 70 post bleach seconds where the formal sensitivity to $P_{\text{initiation}}$ is greatest. To confirm this conclusion, we also plotted the sums of
squares of the errors as a function of $P_{\text{initiation}}$ and found that this function had a minimum in the range of $0.02 < P_{\text{initiation}} < 0.1$.

**Parameter definitions**

Kinetic parameters relevant to RNA pol I dynamics were defined as follows:

- Residence time at promoter = $\frac{1}{(k_{\text{off}} + k_{\text{start}})}$
- Residence time in elongation = $\frac{9}{k_{\text{elong}} + 1/k_{\text{term}}}$
- Residence time in FC = $\frac{1}{(k_{\text{on}} + k_{r})}$
- Residence time in nucleoplasm = $\frac{1}{(k_{r} + k_{\text{nes}})}$
- Residence time in cytoplasm = $\frac{1}{k_{\text{nls}}}$
- Promoter probability = $\frac{k_{\text{on}}}{(k_{\text{on}} + k_{r})}$
- Initiation probability = $\frac{k_{\text{start}}}{(k_{\text{start}} + k_{\text{off}})}$

**Scaling and quantitative assumptions**

The kinetic model describes the behavior of GFP-tagged proteins. Conclusions regarding the behavior of endogenous proteins can be made based on the tracer assumption. Under the experimental conditions, RNA polymerase components in each compartment of the model are contributed from the endogenous pool and the exogenously expressed GFP fusion proteins. Once steady state is established total chemical potentials will remain unchanged even as some fluorescent molecules are bleached, even though there are more copies of the protein in every cellular pool. This means that GFP-tagged proteins serve as tracer molecules in the sense that they are observable, but do not alter the initial steady state (apart from increasing the size of all cellular pools). In a tracer approach, it is possible to scale the cellular pool sizes and fluxes to useful units of
measure. We chose to scale the steady state pool sizes and fluxes to published estimates of the total number of active genes and the number of polymerase molecules per active gene. Based on literature estimates we constrained the total number of Pol I molecules in elongation to be $1.2 \times 10^4$ assuming that the number of active rDNA genes is 100-150 (S4) and the number of polymerases on each active gene is 100-110 (S5, S6). We furthermore assumed an equimolar stoichiometry of RNA pol I. Note, that if the overexpression of proteins has not increased all pools by the same proportion our conclusions regarding in vivo pool sizes and fluxes will not be completely correct. However, the proper distribution of the fusion protein and the absence of any detectable negative effect on rRNA production make it unlikely that our estimates are grossly inaccurate.
Altered FRAP kinetics in transcriptionally silent mitotic cells. FRAP was performed on mitotic CMT3 cells expressing GFP-UBF1 (a), GFP-RPA194 (b), GFP-PAF53 (c) or RPA43-GFP (d) which accumulated in nucleolus organizer regions on mitotic chromosomes. FRAP recovery curves for these elongating factors in mitotic cells show faster recovery rates and the slow component reflecting elongating polymerases is absent from the recovery curve. This effect is not due to altered accessibility of proteins to ribosomal genes as the non-elongating GFP-UBF1 does not show any difference in recovery kinetics between mitotic and interphase cells. Values are averages ± S.D. from at least 10 cells.
**Reorganization of FCs in cells expressing fusion proteins.** CMT3 cells expressing RNA pol I GFP-fusion proteins were treated with DRB for 6h or with Actinomycin D for 3h. DRB treatment induced the dispersion of RNA pol I GFP-fusion proteins, into necklace-like structures scattered throughout the nucleoplasm. Inhibition of RNA transcription by actinomycin D for 3 hours leads to the accumulation of GFP-fusion proteins into several enlarged fibrillar centers of segregated nucleoli. These reorganizations are identical to the ones previously observed for endogenous nucleolar proteins. Bar: 4µm.
### Table S1: Additional parameters extracted from best-fit analysis of iFRAP

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<th>Residence time (s)</th>
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Supplementary References

S1  M. Dundr, T. Misteli, M.O. Olson, J. Cell Biol 150, 433-446
S5  P. Dickinson et al. EMBO J. 9, 2207-2214 (1990)