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#### Supporting Online Material

[www.sciencemag.org/cgi/content/full/298/5598/1616/DC1](http://www.sciencemag.org/cgi/content/full/298/5598/1616/DC1)

Materials and Methods

Fig. S1

Tables S1 to S4

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## Extent of Chromatin Spreading Determined by *roX* RNA Recruitment of MSL Proteins

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The untranslated *roX1* and *roX2* RNAs are components of the *Drosophila* male-specific lethal (MSL) complex, which modifies histones to up-regulate transcription of the male X chromosome. *roX* genes are normally located on the X chromosome, and *roX* transgenes can misdirect the dosage compensation machinery to spread locally on other chromosomes. Here we define MSL protein abundance as a determinant of whether the MSL complex will spread in cis from an autosomal *roX* transgene. The number of expressed *roX* genes in a nucleus was inversely correlated with spreading from *roX* transgenes. We suggest a model in which MSL proteins assemble into active complexes by binding nascent *roX* transcripts. When MSL protein/*roX* RNA ratios are high, assembly will be efficient, and complexes may be completed while still tethered to the DNA template. We propose that this local production of MSL complexes determines the extent of spreading into flanking chromatin.

A key mechanism for regulating eukaryotic gene expression is alteration of DNA packaging into chromatin (1). Modified chromatin architecture can sometimes be propagated long distances in cis from an initiation point (2–6), but the mechanism of such spreading is not understood. The MSL dosage compensation complex is thought to spread along the single male X chromosome in *Drosophila* (7). The MSL complex is composed of at least six proteins and two noncoding *roX* RNAs that paint the male X chromosome, leading to covalent modification of the NH<sub>2</sub>-terminal tails of histones H3 and H4 and twofold hypertranscription of hundreds of linked genes (8–10).

The two *roX* RNAs perform redundant functions (11, 12). The lethality of *roX1 roX2* double-mutant males can be rescued by expression of either *roX1* or *roX2* RNA from autosomal locations, showing that *roX* RNAs can be supplied in trans to coat the X chromosome (12). However, both genes synthe-

sizing *roX* RNAs are normally located on the X chromosome, and we have suggested that this contributes to targeting dosage compensation to the correct chromosome (7).

In certain *msl* mutant backgrounds, the MSL complex is absent from most locations on the X chromosome, but a small subset of sites, termed chromatin entry sites, retain partial complexes (7, 13). Two of these sites are the *roX* genes. When a *roX* gene is moved to an autosome, it recruits MSL complex, which occasionally spreads up to 1 megabase (Mb) into the flanking autosome in a pattern that varies considerably (Fig. 1A). This suggested that the MSL complex recognizes the X chromosome by first binding at *roX* genes (and perhaps additional sites) and then spreading in cis (7). The MSL proteins could recognize the *roX* genes by binding DNA, nascent RNA, or both. MSL proteins bind *roX* RNAs to form active complexes, and each *roX* gene also contains an MSL binding site (9, 14).

The ectopic MSL spreading observed from autosomal *roX* transgenes was seen in only a small fraction of nuclei compared with the invariant MSL pattern in the wild-type male X chromosome (7, 13). During complementation analyses of *roX1 roX2* mutants, we unexpectedly found that the genotype of the X chromosome strongly influenced ectopic MSL spreading from autosomal transgenes. We observed essen-

tially no spreading in the presence of a wild-type X chromosome, but mutations in either *roX1* or *roX2* separately allowed modest MSL spreading from autosomal *roX* transgenes in some nuclei (Table 1; Fig. 1, B to D). In contrast, *roX1 roX2* mutants displayed extensive autosomal MSL spreading [ $>1$  megabase pair (Mbp)] in nearly all nuclei regardless of their insertion site (Fig. 1, E to I; Fig. 2, A and B), including centric heterochromatin (Fig. 1I). In each case, MSL complexes still painted the X chromosome. Autosomal *roX* transgenes were poor sites of MSL spreading if one or both endogenous *roX* genes were functioning on the X chromosome, but the same transgenes supported efficient MSL spreading over autosomes in a *roX1 roX2* double mutant. Thus, *roX* genes appear to compete for limiting components for chromatin spreading.

We next asked if only X-linked *roX* genes could compete with autosomal MSL spreading. We found that a second autosomal *roX* transgene strongly reduced spreading from a reference *roX* transgene. For example, the MSL complex spread several megabase pairs from P{w<sup>+</sup>GMroX2}97F (henceforth transgenics will be referred to as GMroX1-location or GMroX2-location, i.e., GMroX2-97F) in nearly all nuclei when it was the only source of *roX* RNA (Table 1; Fig. 2B). However, spreading was greatly reduced when GMroX1-67B was also present (Fig. 2C; Table 1). We tested seven pairs of *roX* transgenes and found that spreading from one site was reduced in both frequency and extent by the presence of a second *roX* gene (Table 1) (15). This confirms that the factors on the wild-type X chromosome responsible for competing for MSL spreading from an autosomal transgene are the endogenous *roX* genes and shows that *roX* genes are potent inhibitors of ectopic MSL spreading regardless of location.

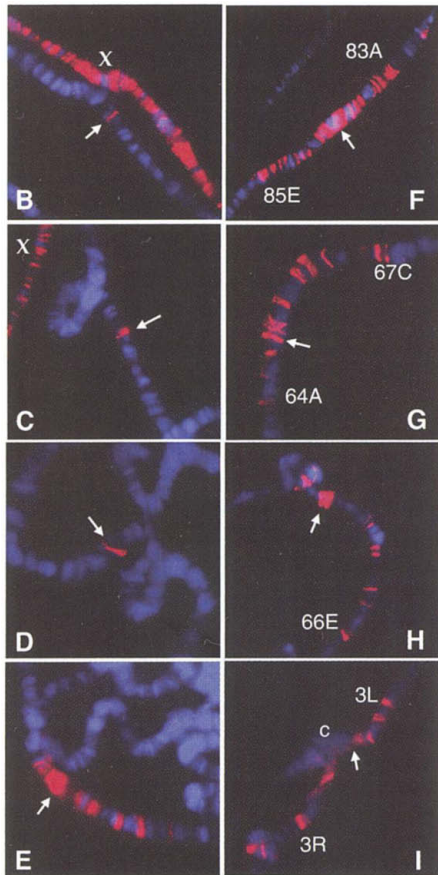
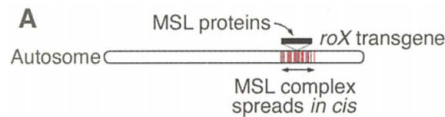
The ability to compete with ectopic MSL spreading might reside in the *roX* RNAs or in the MSL binding sites within the *roX* genes. We constructed stocks in which MSL cis spreading from a reference GMroX2-97F transgene was challenged with two different *roX1* cDNA transgenes, both of which contain an MSL binding site. In one case, the *roX1* cDNA was transcribed from the constitutive *Hsp83* promoter (13). This transgene

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strongly competed with MSL spreading from GMroX2-97F (Table 2). The other *roX1* cDNA transgene lacked a promoter and produced no detectable RNA (Fig. 2D, lane 9)



**Fig. 1.** Extensive spreading of MSL complex from autosomal *roX* transgenes. (A) When a *roX* gene is moved from the X chromosome to an autosome, it recruits the MSL complex to spread in cis from the transgene (7, 13). (B to E) Typical MSL staining (red is antibody to MSL1) in GMroX2-86F males with a wild-type X chromosome (B), *roX1* X chromosome (C), *roX2* X chromosome (D), or *roX1 roX2* double-mutant X chromosome (E). Arrow indicates transgene location. Most nuclei in (B) to (D) have a single MSL band at the autosomal transgene (arrow), but modest MSL spreading can be seen in a low percentage of such nuclei: (B) 4%, (C) 2%, (D) 15%. (F to I) The extent of cis spreading is shown in transgenic *roX1 roX2* males carrying GMroX1-84C (F), GMroX1-65B (G), GMroX1-69C (H), and GMroX1-80C (I). The MSL bands in (I) are seen on either side of the chromocenter (c). The full genotype of the *roX1 roX2* chromosome is *y w roX1<sup>ex6</sup> Df(1)roX2<sup>52</sup> P(w<sup>+</sup>4Δ4.3)*. The *P(w<sup>+</sup>4Δ4.3)* element, needed to supply essential genes lost in *Df(1)roX2<sup>52</sup>* (12), is on the X chromosome at 18F (15).

(14). The nontranscribed cDNA failed to compete for MSL spreading from GMroX2-97F (Table 2). Thus, the active factor competing for ectopic autosomal MSL spreading is the *roX* RNA product. The MSL binding site within *roX1* clearly is not sufficient and may play no role.

One possible explanation for our results is that *roX* transcripts compete to assemble

MSL proteins into functional complexes. Because *roX* RNAs are unstable unless bound by MSL proteins (13, 15), steady-state levels of one *roX* RNA species might fall if another *roX* species captured most MSL proteins. We measured the steady-state level of *roX2* RNA made by GMroX2 when it was the only source of *roX* RNA or when *roX1* RNA was also made from the endogenous gene on the

**Table 1.** *roX* genes compete for MSL spreading. (Upper) Each transgene was placed in four different genetic backgrounds: wild-type X chromosome, *roX1* single mutant, *roX2* single mutant, and *roX1 roX2* double mutant. The percentage of male nuclei showing MSL spreading from the *roX* transgene is given, with the number of nuclei counted in parentheses. The ability of each transgene to restore male viability to *roX1 roX2* males is in the last column (male:female ratio). The MSL spreading observed in *roX1 roX2* usually extended over 1 Mb, while only small clusters of MSL bands around the transgene (<300 kb) spread in other backgrounds. (Lower) All assays were performed in *roX1 roX2* males. Transgenes were tested separately (transgene A only) or in pairs (both A and B) for their ability to support MSL spreading. *N*, number of nuclei examined. Single band, percentage of nuclei in which no MSL spreading occurred at the transgene(s). When autosomal MSL spreading was seen in nuclei carrying two *roX* transgenes, it was much less extensive than when only one transgene was present.

Competition between endogenous <i>roX</i> genes and autosomal <i>roX</i> transgenes					
Transgene	Percentage of nuclei showing MSL spreading				Male viability
	Wild type	<i>roX1</i> <sup>-</sup>	<i>roX2</i> <sup>-</sup>	<i>roX1</i> <sup>-</sup> <i>roX2</i> <sup>-</sup>	<i>roX1</i> <sup>-</sup> <i>roX2</i> <sup>-</sup>
GMroX1-67B	2 (91)	2 (501)	22 (108)	93 (82)	90
GMroX1-85D	0 (99)	16 (94)	3 (134)	100 (279)	45
GMroX1-85E	4 (242)	56 (325)	39 (54)	100 (131)	70
GMroX1-95D	3 (68)	18 (364)	25 (156)	98 (111)	88
GMroX1-102C	0 (142)	0 (130)	20 (181)	100 (162)	82
GMroX2-85B	0 (56)	0 (225)	0 (163)	98 (93)	52
GMroX2-86F	4 (79)	2 (153)	15 (222)	98 (93)	72
GMroX2-97F	0 (72)	3 (231)	3 (169)	97 (114)	76
No transgene					1.5

Competition between pairs of autosomal <i>roX</i> transgenes						
Transgene A	Transgene B	<i>N</i>	Single band	Spreading (%)		
				A only	B only	A and B
GMroX1-82B		299	0	100		
GMroX1-84C		353	0	100		
GMroX1-67B		82	7	93		
GMroX2-85B		93	2	98		
GMroX2-97F		114	3	97		
GMroX1-82B	GMroX1-84C	147	63	30	3	4
GMroX2-85B	GMroX2-97F	76	21	4	68	7
GMroX1-67B	GMroX2-97F	69	62	38	0	0
GMroX1-67B	GMroX2-85B	81	6	89	0	5

**Table 2.** *roX* RNA, not the MSL binding site, competes for MSL spreading. The number of nuclei showing extensive spreading from GMroX2-97F in transgenic *roX1 roX2* males was counted. Small clusters are 2 to 5 closely grouped MSL bands around 97F. Extensive spreading is >5 MSL bands extending more than 1 Mbp from 97F. *N*, number of nuclei counted. Line 1 shows GMroX2-97F alone. Lines 2 and 3 show nontranscribed *roX1* cDNAs at 66C or 85D. Lines 4 and 5 show the *roX1* cDNA transcribed from the *Hsp83* promoter at 61B and 87B. Although MSL complexes bound to *roX1* cDNAs, they showed no detectable spreading in this assay. RNA production in the absence of spreading appeared to be sufficient for competition.

Competing transgene	Nuclei showing MSL spreading (%)			<i>N</i>
	Single band 97F	Small cluster	Extensive spreading	
None	5	13	82	256
Δpro <i>roX1</i> -66C	1	7	92	366
Δpro <i>roX1</i> -85D	18	17	65	410
H83roX1-61B	97	3	0	302
H83roX1-87B	99	1	0	348

X chromosome or an autosomal transgene. When the GMroX2-97F or the GMroX2-85B transgene was the only source of *roX* RNA and complexes spread efficiently in cis, we observed high levels of *roX2* RNA (Fig. 2D, lanes 5 and 7). When *roX1* RNA was also present, MSL spreading was greatly inhibited around the GMroX2 transgenes and the amount of *roX2* RNA fell. It made no difference if the *roX1* RNA came from the endogenous locus on the X chromosome (Fig. 2D, lane 4), the GMroX1 autosomal transgene (Fig. 2D, lane 6), or the H83roX1 transgene (Fig. 2D, lane 8).

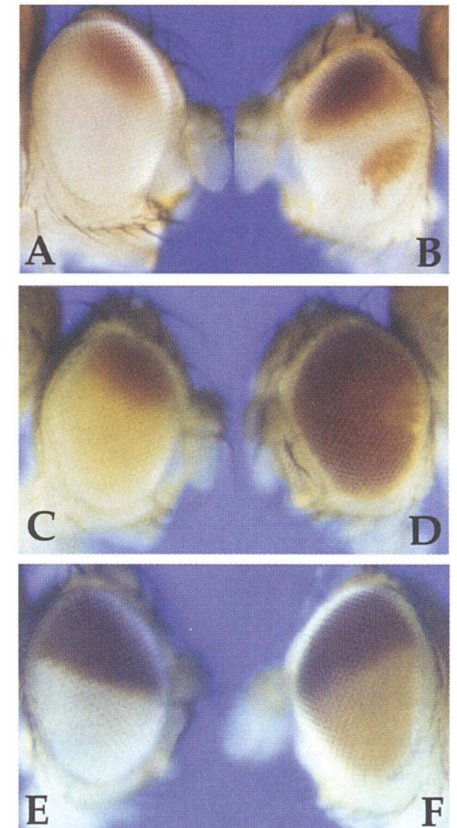
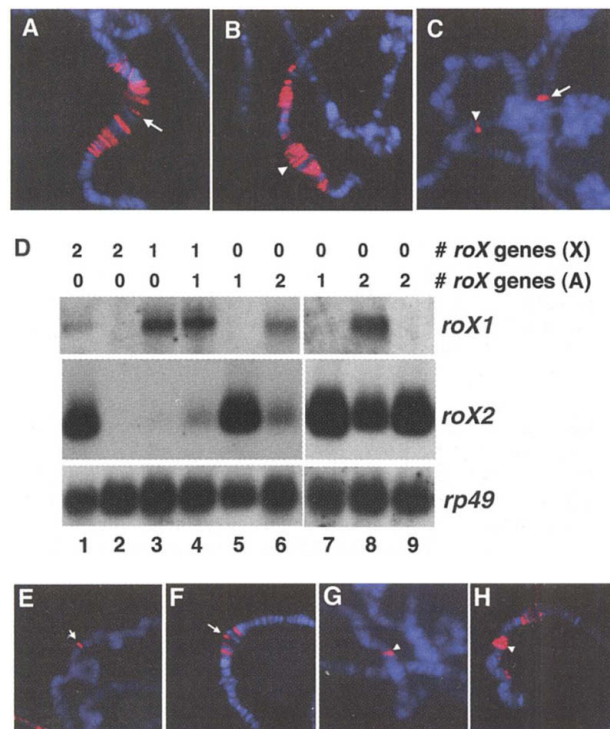
Transcription at GMroX2 might be indirectly repressed by the presence of *roX1* RNA through feedback regulation. Alternatively, similar amounts of *roX2* RNA may be made in all cases, but, when *roX1* RNA competes for MSL proteins, *roX2* transcripts may be inefficiently packaged into complexes and the naked RNAs then degraded. We reexamined ectopic MSL spreading from eight *roX* transgenes under conditions in which both MSL1 and MSL2 were overexpressed from constitutive promoters (16). GMroX1-67B and GMroX2-97F are typical examples that show dramatic spreading in the absence of any other *roX* genes (Fig. 2, A and B) but rare spreading when the X chromosome carried a functional *roX1* or *roX2* gene (Fig. 2, E and G). This competition between *roX* genes was partially overcome when MSL1 and MSL2 proteins were overexpressed, as

spreading from either transgene was clearly increased in both frequency and extent, despite the presence of a functioning *roX* gene on the X chromosome (Fig. 2, F and H) (15). These results strongly suggest that MSL proteins are normally recruited by *roX* RNAs to begin the spreading process and that the local concentration of MSL complexes at a *roX* gene determines the extent of epigenetic spreading into flanking chromatin.

Although the MSL complex has been reported to up-regulate transcription of flanking genes by histone modification (17, 18), this model for dosage compensation in *Drosophila* has been disputed (19). The ability to cause consistent MSL spreading on autosomes provides an opportunity to examine the direct effect of MSL complexes on the transcription status of individual flanking genes. We used a GMroX1 transgene inserted at position 69C, where the linked reporter gene *mini-white* is expressed only in the dorsal part of the eye (Fig. 3, A and B) (20). GMroX1-69C flies showed a striking sex difference in eye pigmentation superimposed on this dorsoventral pattern. Males displayed sporadic red sectors of *mini-white* expression ventrally, suggesting that the *roX* gene recruited the MSL complex to partially overcome the local repressive chromatin environment. When the X chromosome carried a *roX*<sup>+</sup> gene, derepression of *mini-white* was a rare event, resulting in a few pigmented sec-

tors, but in a *roX1 roX2* mutant background, *mini-white* expression in the ventral half of the eye was almost totally derepressed in males (Fig. 3, D versus B and C). As a control, we tested another nearby *mini-white* transgene, P{lacW}<sup>mirr</sup><sup>B1-12</sup>, lacking any *roX* sequences (21), and it was not affected

**Fig. 2.** Pairs of *roX* transgenes compete for limiting MSL proteins. (A to C) Extent of MSL spreading (red) in *roX1 roX2* males with only GMroX1-67B (arrow) (A), only GMroX2-97F (arrowhead) (B), and both transgenes present in the same animal (C). (D) Northern blot showing that RNA made from GMroX2 is abundant when alone but decreases when *roX1* RNA is also made. The same membrane was hybridized to *roX1*, *roX2*, and *rp49* probe as a loading control. The number of endogenous *roX* genes (X) and autosomal transgenes (A) present is indicated above each lane. Lanes: 1, wild-type male; 2, wild-type female; 3 and 4, *roX1*<sup>+</sup> *roX2*<sup>-</sup> males (lane 3, no transgene; lane 4 carries GMroX2-85B); 5 to 9, *roX1 roX2* males (lane 5 carries GMroX2-85B, lane 6 carries both GMroX2-85B and GMroX1-67B, lane 7 has GMroX2-97F, lane 8 has both GMroX2-97F and H83roX1-61B, lane 9 has both GMroX2-97F and the nontranscribed  $\Delta$ pro *roX1*-66C). (E) *y w roX1*<sup>ex6</sup>/Y; GMroX1-67B/+ males show MSL spreading in only 2% of nuclei. (F) Similar males that also overexpress MSL1 and MSL2 show modest MSL spreading in 84% of nuclei. (G) *w Df(1)roX2*<sup>52</sup>/Y; P{w<sup>+</sup> 4 $\Delta$ 4.3}/+; GMroX2-97F/+ males have MSL spreading in only 3% of nuclei. (H) Similar males that overexpress MSL1 and MSL2 show MSL spreading in 45% of nuclei.



**Fig. 3.** MSL spreading visualized by eye color phenotype. *w*<sup>+</sup>GMroX1-69C is inserted in the second intron of the *ara* gene within the *iroquois* cluster (15). The *mini-white* eye color marker shows a typical darker pigment in males than in females in the dorsal eye but is silenced by the local chromatin environment in the ventral half of the eye (20). (A) *y w*; *w*<sup>+</sup>GMroX1-69C/+ females silence *mini-white* in the ventral ~70% of the eye. (B) *y w/Y*; *w*<sup>+</sup>GMroX1-69C/+ males have red sectors in the ventral part of the eye. (C) *y w roX1*<sup>ex6</sup> *Df(1)roX2*<sup>52</sup> P{w<sup>+</sup> 4 $\Delta$ 4.3}; *w*<sup>+</sup>GMroX1-69C/+ females have *mini-white* expression unchanged from *w*<sup>+</sup>GMroX1-69C. Light orange color comes from the P{w<sup>+</sup> 4 $\Delta$ 4.3} transgene that provides essential genes lost in *Df(1)roX2*<sup>52</sup> (12, 15). (D) *y w roX1*<sup>ex6</sup> *Df(1)roX2*<sup>52</sup> P{w<sup>+</sup> 4 $\Delta$ 4.3}/Y; *w*<sup>+</sup>GMroX1-69C/+ males show almost complete derepression of *mini-white* in the ventral half of the eye. (E) *w/Y*; P{lacW}<sup>mirr</sup><sup>B1-12</sup>/TM6, Tb male carries a *mini-white* marked P element about 100 kb from *w*<sup>+</sup>GMroX1-69C and shows similar ventral silencing (21). (F) *y w roX1*<sup>ex6</sup> *Df(1)roX2*<sup>52</sup> P{w<sup>+</sup> 4 $\Delta$ 4.3}/Y; P{lacW}<sup>mirr</sup><sup>B1-12</sup>/+ male shows no derepression of ventral silencing because the B1-12 transgene does not carry a *roX* gene to attract the MSL complex. Light orange ventral eye is due to the P{w<sup>+</sup> 4 $\Delta$ 4.3} transgene. This is a rare escaper male lacking any source of *roX* RNA. About 99% of such males die because of a failure of dosage compensation.

# A Kinetic Framework for a Mammalian RNA Polymerase in Vivo

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by *roX* mutations (Fig. 3, E and F). These results suggest that spreading is likely to be a general phenomenon, occurring in many or most tissues of the animal. When the MSL complex spreads over genes surrounding *roX1*, the chromatin is remodeled for increased transcription. If ectopic MSL spreading occurs in a repressive chromatin environment, even strong silencing may be overcome. The consistency of ectopic spreading correlates with increased transcription of flanking chromatin.

We have found conditions in which *roX* RNAs either assemble into MSL complexes that preferentially spread in cis from the site of transcription or diffuse to the X chromosome. We propose that these outcomes are determined by MSL proteins assembling onto growing *roX* transcripts tethered to the chromosome. The local pool of MSL proteins would control the efficiency of this process. If active complexes are completed by the time 3' RNA processing releases the RNA from the chromosome, the most likely outcome is immediate entry and spreading into flanking chromatin. If the supply of MSL subunits is reduced by a competing source of *roX* RNA, release of *roX* transcripts might precede complex maturation. These MSL complexes are unlikely to return to the transgene after assembly in solution and instead diffuse to the X chromosome, which, in addition to *roX* genes, has unknown features that make it the best target for MSL complexes.

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We have analyzed the kinetics of assembly and elongation of the mammalian RNA polymerase I complex on endogenous ribosomal genes in the nuclei of living cells with the use of in vivo microscopy. We show that components of the RNA polymerase I machinery are brought to ribosomal genes as distinct subunits and that assembly occurs via metastable intermediates. With the use of computational modeling of imaging data, we have determined the in vivo elongation time of the polymerase, and measurements of recruitment and incorporation frequencies show that incorporation of components into the assembling polymerase is inefficient. Our data provide a kinetic and mechanistic framework for the function of a mammalian RNA polymerase in living cells.

Within the mammalian nucleus, the tandemly repeated ribosomal genes are localized specifically in morphologically distinct nucleolar structures termed "fibrillar centers" (FCs), where they are transcribed exclusively by RNA polymerase I (1–3). FCs are naturally occurring gene arrays enriched in components of the RNA pol I machinery and are therefore an ideal system to visualize and to quantitatively study the dynamics of an RNA polymerase on its endogenous target in living cells.

In order to visualize RNA pol I in vivo, we tagged several RNA pol I components, including preinitiation factors Upstream Binding Factor 1 (UBF1), UBF2, and Transcription Associated Factor<sub>48</sub> (TAF<sub>48</sub>), assembly factors Polymerase Associated Factor 53 (PAF53) and Transcription Initiation Factor-1A (TIF-1A/Rn3), and the subunits of the polymerase (RPA194, RPA43, RPA40, and RPA16) with the green fluorescent protein (GFP). The fusion proteins were expressed transiently or stably in CMT3 monkey kidney cells where fusion proteins accumulated in the nucleolus in multiple foci indicative of FCs (Fig. 1, A and B). The punctate sites of accumulation were confirmed to be endogenous ribosomal genes by fluorescence in situ hybridization using a specific probe against the nascent 5' External Transcribed Spacer (ETS) core segment of preribosomal RNA (pre-rRNA) (Fig. 1A) (4). Intact ribosomal

DNA (rDNA) transcription in cells expressing tagged RNA pol I components was confirmed by incorporation of 5-bromouridine 5'-triphosphate (BrUTP) in situ run-on assays (Fig. 1B). As observed for endogenous RNA pol I components by antibody staining, a weak diffuse nucleoplasmic signal and a cytoplasmic pool was detected for all fusion proteins (Fig. 1, A and B) in addition to the strongly labeled FCs. The expected localization of the fusion proteins in nucleolar foci is consistent with their proper functioning, because GFP fusions of several nonfunctional mutants of UBF1, PAF53, and RPA194 did not accumulate in FCs and were found throughout the nucleus (5).

To test whether the GFP fusion proteins were functionally incorporated into the RNA pol I transcription complex, we transfected the fusion proteins into cells stably expressing the FLAG-tagged pol I assembly factor TIF-1A/Rn3 (6). When the RNA pol I holoenzyme was isolated by use of the FLAG epitope, GFP-tagged TAF<sub>48</sub>, TIF-1A/Rn3, PAF53, RPA194, RPA43, RPA40, and RPA16 were recovered with the holoenzyme, whereas GFP-UBF1 and GFP-UBF2 were not efficiently pulled down, confirming their weak association with the holoenzyme (Fig. 1C) (7, 8). Separate experiments confirmed that the pulled-down RNA pol I was transcriptionally active (9).

We used fluorescence recovery after photobleaching (FRAP) to study the dynamics of recruitment of RNA pol I components to endogenous ribosomal genes (Fig. 2) (10). Sites of rDNA transcription in cells expressing one of the RNA pol I fusion proteins were bleached with the use of a short laser pulse that irreversibly

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