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Mammalian cell lines expressing functional RNA polymerase II tagged with the green fluorescent protein

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SUMMARY

RNA polymerase II is a multi-subunit enzyme responsible for transcription of most eukaryotic genes. It associates with other complexes to form enormous multifunctional 'holoenzymes' involved in splicing and polyadenylation. We wished to study these different complexes in living cells, so we generated cell lines expressing the largest, catalytic, subunit of the polymerase tagged with the green fluorescent protein. The tagged enzyme complements a deficiency in tsTM4 cells that have a temperature-sensitive mutation in the largest subunit. Some of the tagged subunit is incorporated into engaged transcription complexes like the wild-type protein; it both resists extraction with sarkosyl and is hyperphosphorylated at its C terminus. Remarkably, subunits bearing such a tag can be incorporated into the active enzyme, despite the size and complexity of the polymerizing complex. Therefore, these cells should prove useful in the analysis of the dynamics of transcription in living cells.

Key words: Complementation, Green fluorescent protein, RNA polymerase, Temperature-sensitive mutant, Transcription

INTRODUCTION

RNA polymerase II (pol II) is a multi-subunit enzyme responsible for the transcription of most genes in higher eukaryotes (Roeder, 1996). It associates with other complexes to form an enormous 'holoenzyme' involved in related functions like splicing, polyadenylation and the repair of damage in DNA (Hampsey and Reinberg, 1999; Holstege and Young, 1999). In turn, several such holoenzymes are organized into even larger structures called transcription 'factories' (Cook, 1999). At the heart of each individual polymerizing complex lies the catalytic subunit; this is the largest subunit found in the core enzyme, and it has homology with the β′ subunit of the bacterial enzyme.

We wished to study the dynamics of the different forms of the polymerase in living cells. Our strategy was to construct a hybrid gene encoding a fluorescently tagged form of the largest subunit of pol II, and to use this gene to correct the genetic defect in a cell line expressing a mutant subunit. tsTM4 is a temperature-sensitive mutant of the CHO-K1 line in which a proline at position 1006 in the largest subunit of pol II is replaced by a serine; it has a complex phenotype, and grows at 34°C but not 39°C (Tsuji et al., 1990; Sugaya et al., 1997). We hoped that introducing the hybrid gene would enable tsTM4 cells to grow at 39°C. Although many mutations/insertions in the largest subunit are viable (Archambault and Friesen, 1993), we feared that adding a large tag might prevent the modified subunit from being integrated into the active polymerizing complex. Therefore, we first tested the smallest tag currently available – the 'FlAsH' tag of six amino acids in an α-helix (Griffin et al., 1998; Tsien and Miyawaki, 1998). Two constructs were made; one (with the tag embedded in a possible α-helix close to the N terminus) yielded no transformants able to grow at 39°C, while the other (with a C-terminal tag) gave transformants in which the hybrid protein was difficult to localize against the background given by the 'FlAsH-EDT_2' reagent (not shown). We therefore tested a larger tag – a modified form of the green fluorescent protein (Tsien, 1998) – attached to the N terminus. To our surprise, many transfectants expressing this tagged subunit were obtained at 39°C. Moreover, the tagged subunit was found in transcription complexes engaged on their templates; it resisted extraction with sarkosyl, and its C-terminal domain (CTD) was hyperphosphorylated like the wild-type protein (Green et al., 1975; Dahmus, 1996). It seems that the subunit bearing such a tag can be incorporated into the active polymerizing complex, despite its size and complexity. As this hybrid protein complements the deficiency, it must function much like the wild-type enzyme. Therefore, these cells should prove useful in the analysis of the dynamics of transcription.

MATERIALS AND METHODS

Isolation of lines expressing EGFP-pol

A plasmid encoding an enhanced version of the green fluorescent

protein (i.e. EGFP) fused with the largest subunit of RNA polymerase II (i.e. hRPB1) was constructed in two main steps. First, the coding sequence of EGFP was flanked by a unique *Avr*II site immediately in front of the ATG and a unique *Nhe*I site replaced the stop codon; both sites were in-frame with the 289 residues in the coding sequence to give the sequence CCT AGG ATG – coding sequence – GAA GCT AGC). A minor change in the coding sequence was achieved by replacing Lys^{289} with an Asp; this does not alter fluorescence. The *Avr*II-*Nhe*I fragment was excised, and inserted into the unique *Nhe*I site of pAT7 (Nguyen et al., 1996), yielding pATGFP with a unique *Nhe*I restriction site at the 3′ end of the coding sequence of EGFP, in-frame with six His codons. In the second step, a 5910 bp *Nhe*I fragment containing the complete hRPB1 coding sequence without a stop codon was inserted in the unique *Nhe*I site of pATGFP. The resulting plasmid encodes a fusion protein with a B10 epitope, EGFP, hRPB1 and six His residues (Fig. 1A). This plasmid was introduced into tsTM4 cells (Tsuji et al., 1990) using the GenePORTER transfection reagent (Gene Therapy Systems, Inc.), and clones able to grow at 39°C were selected. Cells were grown in Ham's F-12 medium plus 10% fetal calf serum and 2 mM L-glutamine for routine culture, or in CO_2-independent medium (Gibco-BRL). For Fig. 1E, 3×10^5 cells in 5 ml were inoculated in 60 mm dishes, and cell numbers counted daily thereafter using a hematocytometer (viable cells were distinguished by their exclusion of Trypan Blue).

Transcription reactions

Conditions used for lysing cells with saponin in a 'physiological' buffer (PB), and for transcriptional 'run-on' reactions ± sarkosyl, [^{32}P]UTP or Br-UTP, have been described (Kimura et al., 1999; Pombo et al., 1999b).

Immunoblotting

Approx. 10^7 cells were washed twice in PB, and divided into four samples; one served as a control and the other three were treated (4°C; 10 minutes) with 0.01% saponin, 0.1% Triton X-100 or 0.1% sarkosyl. Next, treated cells were washed 2× in PB, resuspended in PB, and mixed with an equal volume of 2× sample buffer; then, proteins were resolved on 6% SDS-polyacrylamide gels, and target proteins detected by immunoblotting (Kimura et al., 1999) using the following antibodies: 7C2 (1/10,000 dilution; Besse et al., 1995), H5 (1/500; BabCo), anti-B10 (1/500; Ali et al., 1993). Digital images were collected by scanning the film, and band intensities were measured using ImageQuant (Molecular Dynamics) or Adobe Photoshop software.

Microscopy

Images of cells growing on a heated stage in glass-bottom microwells or chambers (Manders et al., 1999), or of cryosections (Pombo et al., 1999a,b), were collected using a BioRad MRC 1000/1024 hybrid 'confocal' microscope running under Comos 7.0a software (Fig. 1B,C,E; Fig. 3) or a Wallac UltraView 'confocal' microscope (Fig. 1D). Images were incorporated into Photoshop (Adobe) and contrast-stretched to fill the entire gray scale.

RESULTS AND DISCUSSION

tsTM4 is a temperature-sensitive mutant of the CHO-K1 line that grows at 34°C but not 39°C (Tsuji et al., 1990). The deficiency was complemented by introducing a hybrid gene encoding the wild-type form of the largest subunit of RNA polymerase II (pol II) fused with an enhanced version of the green fluorescent protein (EGFP; Tsien, 1998); the hybrid EGFP-polymerase II (EGFP-pol II) was also tagged with the B10 epitope and six histidine residues to facilitate immunolocalization and purification (Fig. 1A). After transfection into tsTM4 cells, many clones able to grow at 39°C were obtained, and 31 were selected for analysis. Different clones expressed different amounts of EGFP detectable by fluorescence microscopy; some contained weakly fluorescing nuclei with almost non-fluorescent cytoplasms (Fig. 1B, C23), while others had brightly fluorescing nuclei and cytoplasms (Fig. 1C, C9). As only a quarter of RNA polymerase in a wild-type cell is engaged (e.g. Kimura et al., 1999), it was likely that most EGFP-pol in such high expressors represented an inactive pool. This proved to be the case, and C23 was selected for intensive study because it had the highest fraction of engaged EGFP-pol (see below).

Confocal microscopy of living C23 cells revealed that the EGFP-pol was concentrated in discrete nucleoplasmic speckles against a diffuse background; little was found in nucleoli (Fig. 1D). During prophase, the hybrid protein progressively left chromatin, so that by the middle of mitosis the condensed chromosomes appeared dark against a bright cytoplasm (Fig. 1E). In some cells, bright speckles could be seen against this background (not shown). These results generally confirm those obtained by indirect immunolabelling of fixed cells (e.g. Wansink et al., 1993; Bregman et al., 1995; Mortillaro et al., 1996; Kim et al., 1997).

The transformant grew much like the wild type. Thus, when wild-type CHO-K1 (grandparent) cells are plated, numbers fall during the first day as cells recover; subsequently they increase slightly faster at 39°C than at 34°C (Fig. 1F, left). The temperature-sensitive parent, tsTM4, grows slightly slower than the wild type at 34°C (Fig. 1F, middle; Tsuji et al., 1990); at 39°C, cell number falls and then increases for 1 day just as before, but then cells die (Fig. 1F, middle). C23 behaves like the wild-type grandparent, both at 34°C and 39°C (Fig. 1F, right). Introducing the hybrid gene corrects the temperature-sensitive phenotype; however, the correction is incomplete, as the population at 39°C contains a slightly higher proportion of dead cells (not shown).

Initially, growth at 39°C has little effect on the temperature-sensitive parent, tsTM4. After the initial fall in cell number, the mutant grows apparently normally for 1 day; only thereafter do cells die (Fig. 1F, middle). This makes it unlikely that exposure to 39°C immediately affects transcription. Indeed, the relative rates of 'run-on' transcription (measured using permeabilized cells) at 34°C and 39°C were roughly similar for the wild-type grandparent, mutant parent and C23 (Fig. 1G). Moreover, 'run-on' activity (measured both at 34°C and 39°C) also declines over several days when tsTM4 cells are switched to the non-permissive temperature (not shown). Clearly, the effects of the mutation only slowly become apparent at 39°C.

We next determined how much EGFP-pol was engaged. The strong detergent sarkosyl disassembles nuclei, strips histones from the template and prevents unengaged polymerases from initiating; however, it leaves >95% engaged pol II, and these polymerases can still 'run-on' along their templates (Green et al., 1975; Jackson et al., 1998). This engaged fraction also contains the largest subunit with a C-terminal domain (CTD) that is hyperphosphorylated (Dahmus, 1996); this form (i.e. form II_O) migrates in a gel with an apparent molecular mass of approx. 240 kDa, compared to its hypophosphorylated counterpart (i.e. form II_A) at approx. 220 kDa. Therefore, engaged pols can be recognized amongst pools of inactive

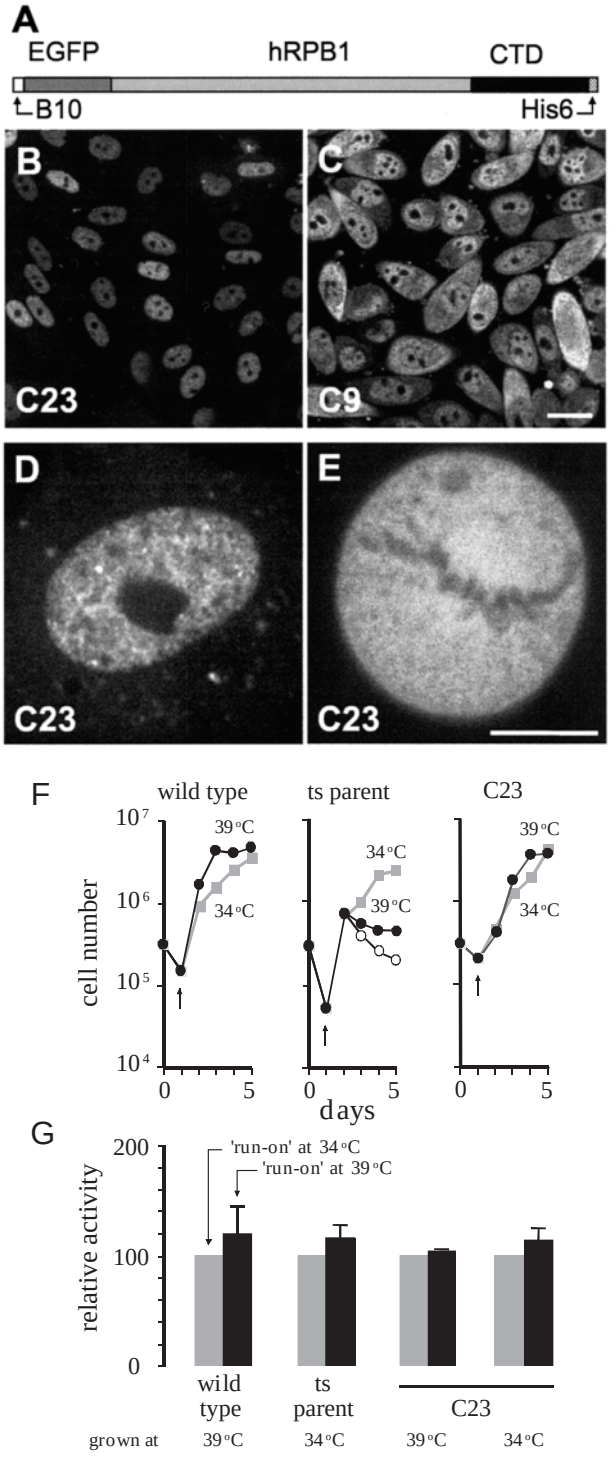

Fig. 1. Properties of cell lines expressing RNA pol II tagged with EGFP. (A) Diagram of the hybrid gene encoding the B10 epitope, EGFP, the largest subunit of pol II (i.e. hRPB1) with its C-terminal domain (CTD), and a His tag. (B,C) Single confocal sections through living cells expressing the above construct; the fluorescent pol in one clone (C23) is mainly nuclear, while another clone (C9) contains higher concentrations in both nucleus and cytoplasm. Bar, 20 μm. (D,E) Single equatorial sections through living interphase (D) or mitotic (E) C23 cells collected using a confocal microscope. Bar, 10 μm. (F) Growth curves of CHO-K1 (wild type), tsTM4 (ts parent) and C23. CHO-K1, tsTM4 and C23 were grown at 34°C, 34°C and 39°C respectively, plated at 34°C, and after 1 day (arrows), dishes were switched to 34°C and 39°C (closed squares and circles respectively). The number of living cells (determined by exclusion of Trypan Blue; open circles) is shown for the ts mutant at 39°C. (G) Cells were grown for >3 days at the temperature indicated, and 'run-on' transcription rates measured at 34°C and 39°C; rates (measured over the first 5 minutes) at 39°C are expressed relative to those at 34°C.

enzyme by their phosphorylation and resistance to extraction with sarkosyl.

We first analyzed proteins in the temperature-sensitive parent, tsTM4. Cells were treated with detergents of increasing strength, washed and residual proteins resolved on a gel; then, the different forms of the largest subunit were detected by immunoblotting using an antibody (i.e. 7C2; Besse et al., 1995) that recognizes both hypo- and hyperphosphorylated forms. Two bands corresponding to forms II_A and II_O are seen in whole cell extracts (Fig. 2A top, lane 1). As with HeLa cells (Jackson et al., 1998; Kimura et al., 1999), treatment with saponin or Triton slightly reduced the intensity of both (lanes 2,3), while sarkosyl removes essentially all form II_A (lane 4). Only the upper band (i.e. form II_O) was detected using an antibody (i.e. H5; Bregman et al., 1995; Kim et al., 1997) that recognizes just the phosphorylated form (Fig. 2A middle, lane 1), and some of this band resisted sarkosyl (lane 4). An antibody (Ali et al., 1993) directed against the B10 epitope that is not present in these cells gives no signal (Fig. 2A bottom, lanes 1-4). CHO-K1 gave blots much like tsTM4 (not shown).

Equivalent loadings of whole-cell extracts of C23 grown at 39°C give four bands with the antibody that recognized both hypo- and hyperphosphorylated forms (Fig. 2A top, lane 5). Two were encoded by the endogenous gene and were present in the amounts seen with the temperature-sensitive parent (results from underexposure, not shown); two were encoded by the transfecting gene and were so intense that they obscured the other two. Treatment with saponin or Triton again reduced the intensities of all four bands (lanes 6,7), while sarkosyl left some EGFP-pol II_O and background levels of EGFP-pol II_A (lane 8). An antibody directed against the phosphorylated form of the CTD confirmed that the main hyperphosphorylated form in the transformant gave the most intense band, and this was the main one that resisted sarkosyl (Fig. 2A middle, lanes 5-8). An antibody directed against the B10 epitope recognized only the two bands encoded by the transfecting gene, and only the hyperphosphorylated form remained after extraction with sarkosyl (Fig. 2A bottom, lanes 5-8). These results suggest that C23 contains large pools of EGFP-pol in addition to the temperature-sensitive enzyme, and that a significant fraction of this EGFP-pol is engaged. Similar results were obtained over a period of 6 months (not shown). Measurement of expression levels (by quantitative immunoblotting) in the other 30 clones revealed considerable clone-to-clone variation (Table 1); clone 23 was selected for the most intense study because it contained the highest fraction of EGFP-pol II_O and only average amounts of EGFP-pol II_A. Thus, the sarkosyl-resistant fraction of EGFP-pol II_O represents approx. 10% of all polymerases in the cell but >70% engaged pol II_O (Table 1).

We next determined if the fraction resistant to sarkosyl could 'run-on' along the template. Cells were permeabilized with saponin, preincubated with 0.1 μg/ml actinomycin D to inhibit polymerase I (Chambon, 1974), sarkosyl added, and initial

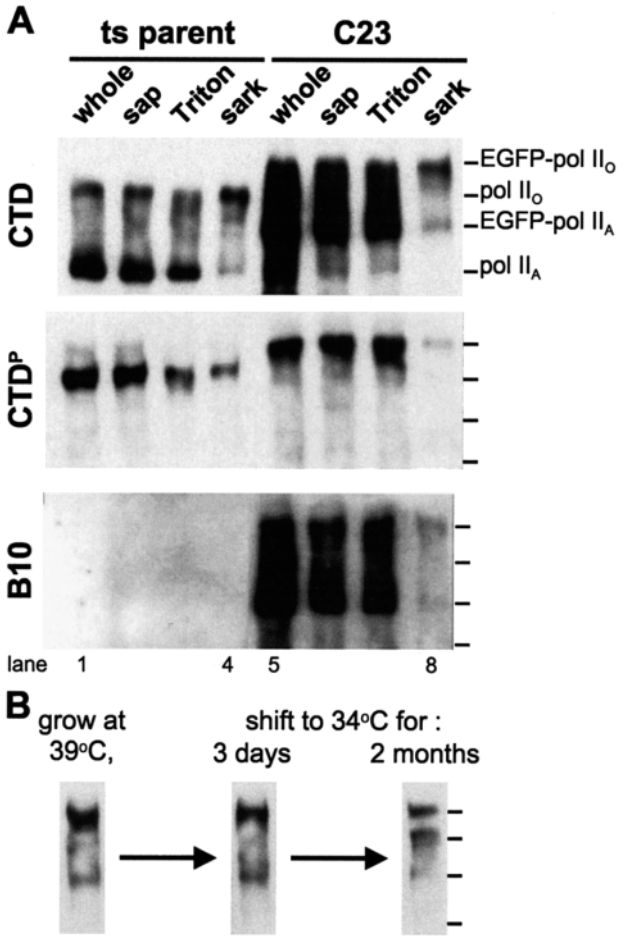

Fig. 2. Some EGFP-pol in C23 is engaged. (A) tsTM4 (ts mutant) and C23 were grown at 34°C and 39°C, respectively, treated ± different detergents, washed, the remaining proteins resolved on an SDS-gel, and different forms of the largest subunit of polymerase II detected by immunoblotting using antibodies directed against the CTD, the phosphorylated form of the CTD and the B10 epitope; only relevant parts of the blot are shown. Positions of unphosphorylated and phosphorylated forms (i.e. forms II_A and II_O) of endogenous and hybrid pols are shown. (B) Different pols (detected as in A) in C23 cells grown at 39°C (left), and after switching to 34°C for 3 days (middle) or 2 months (right). Samples were treated with Sarkosyl.

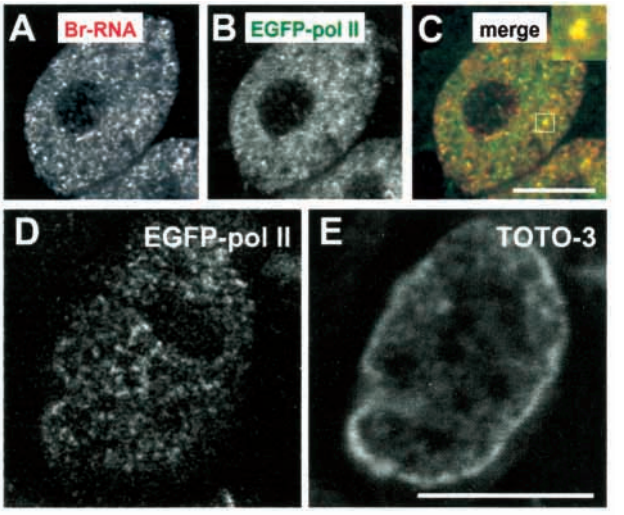

Fig. 3. Some sites rich in EGFP-pol II are transcriptionally active. Permeabilized C23 were allowed to extend nascent transcripts in Br-UTP, fixed, the resulting Br-RNA immunolabelled with Cy3, and three views through the centre of a cell collected using a conventional confocal microscope. (A) Nascent Br-RNA is found in many discrete nucleoplasmic sites, and in some sites in nucleoli. (B) EGFP-pol II gives an even more complex pattern; again, little is found in nucleoli. (C) Merge of images in A and B; inset illustrates an enlargement of the boxed region. Many EGFP-pol II foci are transcriptionally active, and appear yellow. Bar, 10 μm. (D,E) Two views of one cryosection of approx. 100 nm (counterstained with TOTO-3) collected using a confocal microscope. Bar, 10 μm.

rates of 'run-on' transcription measured at 34°C. When grown and assayed at 34°C, polymerases in clone 23 transcribed at 101% of the rate given by the temperature-sensitive parent and, when grown and assayed at 39°C, they transcribed at 110% of the rate given by wild-type grandparent (not shown).

Clearly, the tagged enzyme initiates more effectively at the non-permissive temperature than its temperature-sensitive counterpart present in the same cell. Can it do so at the permissive temperature? To answer this question, cells were grown at 39°C, switched to 34°C, and the sarkosyl-resistant forms detected as before. 3 days later, the pattern remained unchanged, but after 2 months the fraction of endogenous pol II_O had increased (Fig. 2B), and this pattern persisted for >6 months (not shown). This shows that while EGFP-pol is less competitive in the long term, it still remains responsible for half the transcription in the cell. This level, however, is probably only achieved because the pool of inactive enzyme is fourfold higher (Table 1).

Table 1. Expression levels of different forms of polymerase II

	Relative intensity ± s.d. (range)				
	ts parent[a]		31 transfectants	clone 23[a]	
Form of polymerase II	− sarkosyl	+ sarkosyl	− sarkosyl	− sarkosyl	+ sarkosyl
EGFP-pol II_O			1.1±0.7 (0.1-3.0)	3.0±0.5	1.3±0.3
pol II_O	0.7±0.3	0.5±0.7	1.5±0.8 (0.4-4.5)	2.9±0.5[b]	0.5±0.1[b]
EGFP-pol II_A			3.9±2.8 (2.0-15)	3.8±0.5	0.4±0.2
pol II_A	1	≤0.1	1	1	≤0.1

Images like those in Fig. 2A were prepared using the 7C2 antibody; then, band intensities were measured, and expressed relative to those of form II_A in the same cell. tsTM4, the 31 transfectants, and clone 23 were grown at 34°C, 39°C and 39°C, respectively.

[a]Results from ≥3 experiments using several exposures in each to optimize the intensities of individual bands.

[b]Overestimate, as band contains significant contamination from adjacent ones.

In fixed cells, immunolabelling reveals that the endogenous pol is concentrated in numerous speckles in the nucleoplasm above a diffuse background; only a fraction of these speckles are transcriptionally active (Wansink et al., 1993; Bregman et al., 1995; Mortillaro et al., 1996; Kim et al., 1997). We demonstrated that this was also the case in the transformant. Permeabilized cells were allowed to extend nascent transcripts in Br-UTP, fixed, and the resulting Br-RNA immunolabelled with Cy3. Nascent Br-RNA was concentrated in many discrete nucleoplasmic sites (Fig. 3A), and some of these also contained EGFP-pol II (Fig. 3B) to give yellow in the resulting merge (Fig. 3C). Data in Table 1 suggest 70% of this Br-RNA was made by EGFP-pol II.

The resolution of a confocal microscope is limited to approx. 200 nm in the *x* and *y* axes, and ≥500 nm in the *z* axis (Pawley, 1995). As a result, images like those shown in Fig. 3B contain fluorescence from above and below the optical plane. A simple way of improving *z*-axis resolution is to analyze thin physical sections of approx. 100 nm (Pombo et al., 1999a). In such a section that has been counterstained with TOTO-3, EGFP-pol is seen to be concentrated in euchromatic regions of the nucleoplasm, but not in nucleoli or heterochromatin (Fig. 3D,E). This pattern is more complicated than that given by nascent Br-RNA in similar cryosections (Pombo et al., 1999a,b), presumably because it includes fluorescence from unengaged polymerases.

These results show that these stable transfectants contain both temperature-sensitive pol and EGFP-pol, and that the tagged form in one transfectant behaves much like the endogenous enzyme. Thus, C23 cells grow at 39°C like wild-type grandparental cells (Fig. 1F), their overall transcription rate is similar (Fig. 1G), and the tagged enzyme is distributed in and around transcription factories like the endogenous enzyme (Fig. 3). Moreover, EGFP-pol constitutes more than 70% of the engaged polymerase in the cell at 39°C (Table 1), and some hybrid protein even remains competitive at 34°C (Fig. 2B). The hybrid gene (Fig. 1A) also complements the temperature-sensitive defect in a BHK mutant, tsAF8 (Meiss and Basilico, 1972; not shown). It seems that this catalytic subunit carrying a tag at the amino terminus can be incorporated into the core enzyme, and so into the holoenzyme and transcription factory, without much effect on function. Therefore, these stable lines should prove useful in the analysis of the dynamics of transcription.

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