EXPERIMENTAL CELL RESEARCH 313 (2007) 3026–3033

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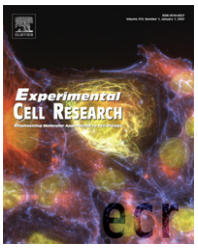

Research Article

Photobleaching reveals complex effects of inhibitors on transcribing RNA polymerase II in living cells

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ARTICLE INFORMATION

Article Chronology:
Received 15 February 2007
Revised version received
25 April 2007
Accepted 27 April 2007
Available online 18 May 2007

Keywords:
Fluorescence loss in photobleaching
Green fluorescent protein
RNA polymerase II
Transcription
Transcriptional inhibitors

ABSTRACT

RNA polymerase II transcribes most eukaryotic genes. Photobleaching studies have revealed that living Chinese hamster ovary cells expressing the catalytic subunit of the polymerase tagged with the green fluorescent protein contain a large rapidly exchanging pool of enzyme, plus a smaller engaged fraction; genetic complementation shows this tagged polymerase to be fully functional. We investigated how transcriptional inhibitors – some of which are used therapeutically – affect the engaged fraction in living cells using fluorescence loss in photobleaching; all were used at concentrations that have reversible effects. Various kinase inhibitors (roscovitine, DRB, KM05283, alsterpaullone, isoquinolinesulfonamide derivatives H-7, H-8, H-89, H-9), proteasomal inhibitors (lactacystin, MG132), and an anti-tumour agent (cisplatin) all reduced the engaged fraction; an intercalator (actinomycin D), two histone deacetylase inhibitors (trichostatin A, sodium butyrate), and irradiation with ultra-violet light all increased it. The polymerase proves to be both a sensitive sensor and effector of the response to these inhibitors.

Introduction

In order to study the dynamics of RNA polymerase II in living cells, we developed a cell line expressing the largest (catalytic) subunit, RPB1, tagged with the green fluorescent protein (GFP). Our cell line, C23, is derived from tsTM4, a temperature-sensitive mutant of the Chinese hamster ovary cell, CHO-K1. The mutation in tsTM4, which grows at 34 °C but not at 39 °C, has been mapped to RPB1 [1,2]. The gene encoding wild-type human RPB1 was fused with another encoding GFP, and the construct expressed in tsTM4; the resulting GFP-tagged polymerase (GFP-pol) complemented the defect at the restrictive temperature (39 °C), and so enabled normal growth [3,4]. This indicates the tagged polymerase is functional at 39 °C, as C23 cells depend on it for survival. However, C23 also contains the original temperature-sensitive (endogenous) enzyme that is used in conjunction with the GFP-pol at 34 °C, and to a lesser extent at 39 °C. We have applied various photobleaching techniques – including FRAP (fluorescent recovery after photobleaching) and FLIP (fluorescence loss in photobleaching) – to analyze the kinetics of the tagged polymerases in this cell line [5,6]; others have also analyzed kinetics using related constructs [[7–9]; reviewed by [10,11]]. All studies indicate there are two major kinetic fractions of polymerase II, with ~75% forming a rapidly exchanging pool and ~25% temporarily immobilized on the template, presumably because it is engaged.

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doi:10.1016/j.yexcr.2007.04.036

The carboxy-terminal domain (CTD) of the largest catalytic subunit of RNA polymerase II plays an important role in regulating mRNA production [12]. In mammals, this CTD contains 52 repeats with the consensus sequence YSPTSPS, which is reversibly phosphorylated *in vivo* at serines 2 and 5 (Ser2 and Ser5). On transcriptional initiation, the cyclin-dependent kinase (CDK)-7 subunit of TFIIH phosphorylates Ser5 [13], so the CTD can activate factors that cap the 5′ end of the nascent RNA [14]. Subsequently, the positive transcription elongation factor, P-TEFb – which contains CDK9 and a cyclin T subunit – phosphorylates Ser2; as a result, phospho-Ser2 is widely used as a marker of productive elongation [15]. Phosphorylation of the CTD also activates splicing and cleavage/polyadenylation [16,17], and splicing recruits P-TEFb to the transcribing polymerase – and in turn this promotes elongation [18]. Moreover, recognition of the polyadenylation signal plays a central role in termination of transcription, which may involve loss of CTD phosphorylation [19]. Therefore, the CTD is cyclically phosphorylated and dephosphorylated during transcription and the associated processing of the transcript.

Not surprisingly, various steps during transcript production can be inhibited by kinase inhibitors [20–23]. These inhibitors include DRB (dichloro-1-β-D-ribofuranosylbenzimidazole), KM05283 (8-(methylthio)-4,5-dihydrothieno[3,4-*g*][1,2]benzisoxazole-6-carboxamide), H-7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine, 2HCl), H-8 (*N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide HCl), H-9 (*N*-(2-aminoethyl)-5-isoquinolinesulfonamide, HCl), H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide, 2HCl), roscovitine (2-(*R*)-1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine), and alsterpaullone (9-nitro-7,12-dihydroindolo-[3,2-*d*][1]benzazepin-6(5*H*)-one). For example, DRB, KM05283, H-7, and H-8 all affect Ser2 phosphorylation, and inhibit 3′ end formation of transcripts from U2 snRNA genes [15,24–27], while roscovitine inhibits phosphorylation of the CTD and so RNA synthesis [28]. Microarray studies also show that DRB rapidly reduces steady-state levels of a wide range of transcripts, and that roscovitine also has complex effects on gene expression profiles [29].

Therefore, we analyzed the effects of these inhibitors on the kinetics of RNA polymerase in living cells. We also tested various other inhibitors. Actinomycin D intercalates into DNA, and arrests transcribing polymerase II [5]; studies using microarrays show steady state levels of transcripts copied from most genes fall rapidly [29,30]. The polymerase also arrests at the intrastrand cross-links induced by cisplatin (*cis*-diammineplatinum(II) dichloride [31,32]), and is then ubiquitinated and degraded [33]; microarrays again show that the effects are general without changing the relative abundance of many mature transcripts [34]. The role that ubiquitinylation and degradation plays in the recovery from damage prompted us to analyze the effects of two compounds – MG132 and lactacystin – that inhibit degradation by the proteasome [35]. These inhibitors do not seem to affect the polymerase directly, but they do prevent its UV-induced ubiquitinylation and loss [36]; they also change the relative abundance of few mature transcripts [37,38]. We also examined two histone deacetylase (HDAC) inhibitors – trichostatin (TSA) and sodium butyrate [39,40]; these induce histone hyperacetylation – which is a general marker for gene activity – although they again have little affect on the relative abundance of most mature transcripts [41,42].

Methods

Cells and drugs

C23 cells, a clonal derivative of tsTM4 cells expressing the largest subunit of polymerase II (RPB1) tagged with GFP under the control of the cytomegalovirus promoter [3], were grown at 39 °C in Ham's F-12 medium (Invitrogen Ltd, Paisley, UK) plus 10% fetal calf serum.

The following drugs were used (all from Sigma-Aldrich, Poole, Dorset, UK, unless stated otherwise): sodium butyrate at 5 mM, DRB (Merck Biosciences, Beeston, Notts, UK) and KM05283 (Maybridge Trevillett, Tintagel, Cornwall, UK) at 100 μM, alsterpaullone (A.G. Scientific, San Diego, CA, USA) and roscovitine (A.G. Scientific) and H-7 (Merck Biosciences) and H-8 and H-89 and H-9 (Merck Biosciences) and TSA (Biomol International, Exeter, UK) all at 50 μM, cisplatin at 25 μM, lactacystin at 20 μM, MG132 (Merck Biosciences) at 10 μM, and actinomycin D at 5 μg/ml.

FLIP

C23 cells expresses variable amounts of GFP-pol in the cytoplasm, but more constant amounts in the nucleus; cells expressing low cytoplasmic levels were selected for study to minimize any contribution of nuclear import to fluorescent recovery. [Such a contribution must be small, as repeated photobleaching of a small nuclear region over several seconds had little effect on cytoplasmic fluorescence.]

Cells were grown in glass-bottomed microwell dishes (Mat Tek, Ashland, MA) for 40–48 h to 50% confluence and imaged at 39 °C on the microscope stage. FLIP experiments were performed as described previously [5,43] using a Radiance 2000 confocal microscope (488 nm laser line; 25 mW argon laser at 6% power; 8× zoom, gain 31, scan speed 600 lines/s; detection using LP500 filter and pinhole aperture 10; image size 256×256 pixels where 1 pixel=105 nm; BioRad Laboratories, Hemel Hampstead, UK) fitted on a TE300 microscope (Nikon UK Limited, Kingston-upon-Thames, Surrey, UK) and a 60× PlanApo objective (numerical aperture 1.4). A field with two cells was selected, imaged every ~0.43 s for 14 s, and the bottom half of one nucleus bleached with 100% laser power as the field was scanned every 0.43 s for another 130 s. The intensity of the unbleached half of the bleached nucleus was measured and normalized relative to the unbleached nucleus. Relative intensities in the bleached area were measured and normalized using the average intensity before bleaching. On one day, 7–15 cells exposed to a drug for 1–2 h – plus an equivalent number of untreated cells – were analyzed; such experiments were repeated ≥3 times. Alternatively, medium was removed from cells, the cells exposed to a UV-light (Sylvania germicidal tube; dose rate of UV-C of 1 J/m^2/s) and regrown in medium for 1 h, and FLIP performed over the next hour. The significance of differences seen between cell populations was determined using GraphPad Prism4 software v4.03 (http://www.graphpad.com) and the two-tailed Mann–Whitney test.

Transcriptional activity determined using bromo-uridine

Transcription rates were determined using bromo-uridine (BrU; [44]). Each experiment involved cells from 4 dishes, 2 treated without and 2 with a drug; each experiment was repeated on ≥3 different days. Cells were grown ± drug for 1 or 2 h (to assess the extent of the inhibition of transcription), or for 2 h before cells were washed and regrown for 24 h in the absence of the drug (to assess recovery from inhibition). 2.5 mM BrU (Sigma-Aldrich) was now added to the medium, and cells grown for a further 15 min to allow engaged polymerases to extend their transcripts; after fixation (20 min; 4% paraformaldehyde in 250 mM HEPES; pH 8.0), the resulting Br-RNA was indirectly immunolabelled with Cy3 using a primary mouse monoclonal anti-BrdU (1/50 dilution of BMC-9318 antibody that also recognizes BrU; Roche Diagnostics Ltd, Lewes, East Sussex, UK) followed by a secondary Cy3-conjugated donkey anti-mouse IgG antibody (1:200 dilution; Jackson Immunoresearch, West Grove, PA, USA). After counterstaining DNA with Hoechst 33342 (100 ng/ml; Sigma-Aldrich), images of duplicate sets of 4 fields of ~50 cells from one dish were collected using a CCD camera attached to a Zeiss Axioplan 2e fluorescence microscope (63× objective; numerical aperture 1.4). Images were analyzed automatically using scripts written with 'Definiens Developer' software (Munich, Germany). Information in images was segmented and classified using intensity, contrast, and shape information, to generate a hierarchical network of interlinked objects. Nuclei were first defined using Hoechst fluorescence, before background, cytoplasmic, and nucleolar objects were detected using GFP fluorescence; the nucleoplasmic area was then defined by subtracting nucleolar area from nuclear area. Finally, the intensity of Cy3 fluorescence in the nucleoplasm was measured, and Cy3 fluorescence in non-cellular background was subtracted.

Results

An example: H-7

Fig. 1 illustrates a typical FLIP experiment used to determine the effect of H-7 on the kinetics of GFP-pol in living C23 cells. H-7 inhibits various kinases including protein kinases A, C, II, and casein kinase I; it also inhibits phosphorylation of Ser2 in the CTD of RNA polymerase II, and the processing of the 3′ ends of some of its transcripts [25]. Cells were incubated with the drug for 1 h, before FLIP experiments were carried out on ~10 different cells (in the continued presence of the drug) over the next hour. The drug concentration was chosen to be sufficient to inhibit transcription significantly within 1–2 h, but insufficient to prevent complete recovery of transcription during subsequent growth in the absence of drug (below). For each FLIP experiment, a field containing two nuclei was selected and imaged (Fig. 1A), and then re-imaged every 0.43 s for 14 s to establish a baseline. As the next image was collected (at $t=0$ s), laser power was increased as the laser scanned through a rectangle containing the bottom half of the lower (target) nucleus; this partially bleaches GFP-pol in the target area. Unfortunately, the resulting power fluctuations

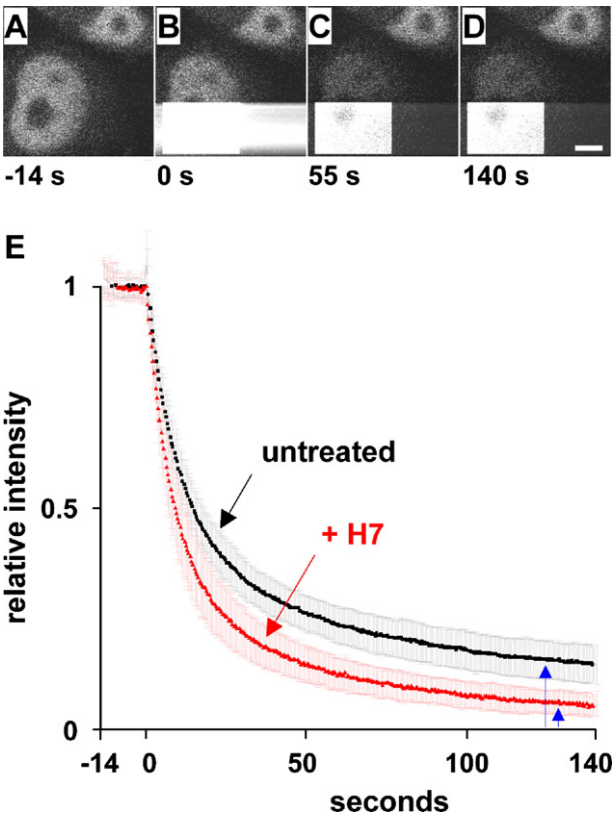

Fig. 1 – An example of a FLIP experiment, illustrated using the kinase inhibitor, H-7. (A–D) C23 cells were incubated in 100 μM H-7 for 1–2 h (in this case, for 1.25 h), and an image of a typical equatorial confocal section collected (at $t=-14$ s); bright fluorescence marks the presence of GFP-pol. Similar images were now collected every 0.43 s for 14 s to establish a baseline. As the next image was collected (at $t=0$), laser power was increased as the laser scanned the white rectangle covering the lower part of the lower nucleus; this partially bleaches GFP-pol in the target area. The combined imaging/bleaching was now continued every ~0.43 s for a further 140 s until most fluorescence disappeared from the top half of the (bottom) target nucleus; typical images obtained after 55 and 140 s are shown. The upper nucleus is used to monitor the small amount of bleaching that occurs during imaging. The signal to the left and right of the bleached area is an artifact and does not affect measurements in the top half of the nucleus. After 55 s, fluorescence in the top half of the bottom (target) nucleus has fallen substantially relative to that seen at −14 and 0 s, and after 140 s it has fallen only a little further. Bar: 5 μm. (E) Quantitative analysis. The average relative intensity (±SD) seen in the top (unbleached) half of 26 target nuclei like the one shown in A–D is given in red; results were collected from 3 different days. Similar results are given for untreated cells. H-7 decreases relative intensities, and the scale of the reduction (difference between blue arrows) is given by the difference between values seen at 130 s (obtained by averaging relative intensities between 120 and 140 s).

and delayed recovery from saturation of the photomultiplier degrade the image to the left and right of the rectangle (Fig. 1B). This combined imaging/bleaching was now repeated every ~0.43 s for another 140 s until most fluorescence disappeared from the top half of the target nucleus (Figs. 1C, D). The intensity in the unbleached half of the target nucleus was then expressed relative to its original (unbleached) intensity, and the value further corrected for the slight effects of bleaching during imaging (using the small reduction in fluorescence seen in the upper, unbleached, nucleus). Relative intensities obtained at each time point with three or more sets of ~10 cells measured on different days were then averaged, and results are given by the red line in Fig. 1E. The relative intensity remains constant until bleaching begins (at $t=0$); it then declines rapidly at first, and then more slowly. The decline seen with untreated cells is not so significant (Fig. 1E, black line).

The curves in Fig. 1E are interpreted as follows. If all GFP-pol diffused freely, bleaching the bottom half of the target nucleus would progressively reduce the relative intensity in the top half to zero because unbleached molecules could diffuse into the rectangle and be bleached; this is the result obtained with control cells expressing free GFP [5]. On the other hand, if all GFP-pol were engaged and so unable to diffuse, the relative intensity would remain at unity because immobile molecules in the top half could never enter the rectangle to be bleached; this is seen with fixed cells [5]. Results lie in between, and are consistent with the existence of two (or more) pools. The largest exchanges rapidly while the smaller one is relatively immobile, representing a large free population (which might contain a fraction that is transiently bound to DNA) and a smaller engaged one; as engaged polymerases terminate, they enter the larger rapidly exchanging pool to be bleached and the relative intensity slowly declines [5]. [Additional, smaller, pools may also exist [6].] Comparison of the curves obtained with and without H-7 shows the drug increases the size of the rapidly exchanging pool at the expense of the engaged fraction. We conclude that H-7 inhibits one or more of the kinases required for initiation and/or productive elongation, consistent with the results obtained *in vitro* (above).

A rough estimate of the scale of the effect of H-7 is obtained as follows. We assume that essentially all the rapidly exchanging pool initially present is bleached by 130 s, with the remaining intensity representing engaged enzyme; then, the difference between the two blue arrows in Fig. 1E reflects the extent to which the drug reduces the engaged fraction. In this case, H-7 reduces the engaged GFP-pol to 40% of the control (Table 1).

Table 1 – Effects of drugs on the fraction of engaged polymerase II measured using FLIP and BrU incorporation

Drug	FLIP		BrU incorporation (%±SD)	
	Relative intensity at 130 s (±SD)	Engaged GFP-pol (%)		
Exposure	1–2 h		1 h	2 h
Untreated	0.15±0.04	100	100	100
Kinase inhibitor				
H-8	0.10±0.04	69	81±5	73±11
H-89	0.09±0.04	63	55±15	31±4
H-9	0.08±0.04	52	56±5	46±4
H-7	0.06±0.02	40	38±3	28±3
Roscovitine	0.06±0.04	38	27±1	25±5
DRB	0.04±0.03	27	37±2	24±2
KM05283	0.03±0.02	19	35±1	22±1[a]
Alsterpaullone	0.02±0.02	17	25±4	21±2
Intercalator				
Actinomycin D	0.2±0.04	136	45±2	28±2[a]
Cross-linker				
Cisplatin	0.08±0.04	52	85±12	49±7
Proteasome inhibitor				
Lactacystin	0.11±0.04	75	81±15	79±10
MG132	0.1±0.03	67	64±11	58±5
HDAC inhibitor				
TSA	0.16±0.07	111	68±10	65±3[a]
Sodium butyrate	0.16±0.04	107	96±15	74±4
UV light				
5 J/m^2	0.17±0.06[b]	179[b]	40±8	35±5[a]
10 J/m^2	0.22±0.05[b]	230[b]	22±5	16±5[a]
20 J/m^2	0.23±0.06[b]	241[b]	16±4	12±1[a]

Relative intensities at 130 s were determined from experiments like those illustrated in Fig. 1E by linear interpolation of values between 120 and 140 s, and expressed as a percentage of untreated controls. These percentages reflect the fraction of engaged GFP-pol. All relative intensities (at 130 s) given by treated samples were significantly different from untreated controls ($p<0.005$; values determined using the two-tailed Mann–Whitney test), except for TSA and sodium butyrate, which were marginally significant ($p=0.03$). Nucleoplasmic signal intensities obtained after growth in BrU were determined from ≥3 independent experiments like that illustrated in Fig. 4, and expressed as a percentage of those given by untreated controls. After 2 h of growth in the presence of the drug (or irradiation with UV light with the dose indicated) followed by 24 h in its absence, BrU incorporation recovered to >90% untreated controls except for KM05283, actinomycin D, TSA, and UV doses of 5, 10, and 20 J/m^2 (which recovered to 40±2%, 77±12%, 81±10%, 78±6%, 56±15%, and 28±5%, respectively).

[a] Indicates such partial recovery.

[b] See legend to Fig. 3.

Kinase inhibitors

We next tested other kinase inhibitors. H-8, H-89, H-9, DRB, alsterpaullone, roscovitine, and KM05283 all had roughly similar effects as H-7 (Figs. 2A, B); they increased the rapidly exchanging pool at the expense of the engaged fraction. The results obtained with DRB confirm those found earlier [5,6]. As we shall see, the extent of the reduction in the engaged polymerase correlates roughly with the extent of transcriptional inhibition (Table 1).

Actinomycin D and cisplatin

Actinomycin D intercalates into DNA, arrests transcribing polymerase II, and increases the fraction of immobile polymerase [5] – unlike the kinase inhibitors; we confirmed this here (Fig. 2C). In contrast, another drug that generates covalently linked adduct and also arrests the polymerase *in vitro* – cisplatin – reduces the engaged fraction (Fig. 2C). This is consistent with release of the stalled polymerase from the template (probably involving the transcription-coupled repair pathway and proteolytic degradation), and/or a reduced

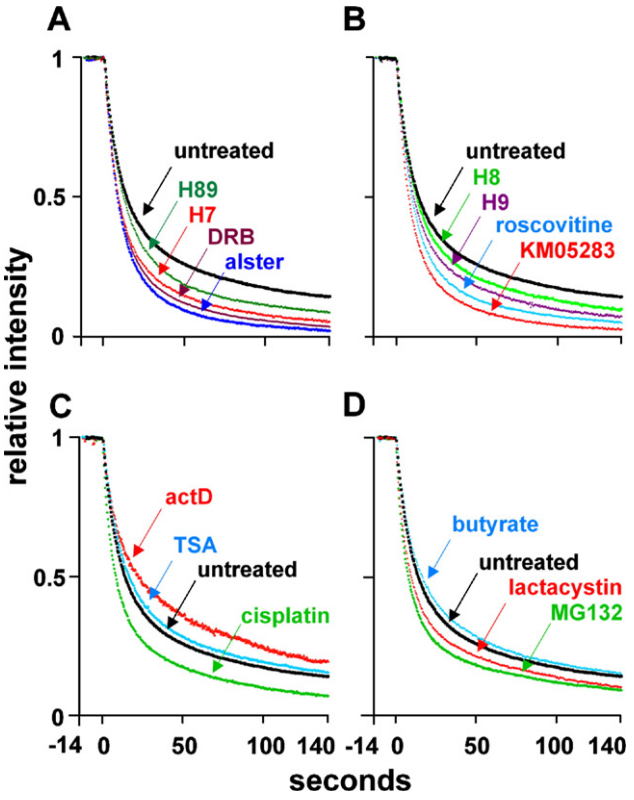

Fig. 2 – GFP-pol kinetics analyzed (using FLIP as in Fig. 1) in cells ($n \geq 25$) exposed for 1–2 h ± various drugs. Drug concentrations: sodium butyrate at 5 mM; DRB and KM05283 at 100 μM; alsterpaullone, roscovitine, H-7, H-8, H-89, H-9, and TSA all at 50 μM, cisplatin at 25 μM, lactacystin at 20 μM, MG132 at 10 μM, and actinomycin D at 5 μg/ml. SD values (not shown) were generally in the range shown in Fig. 1B and all curves given by the drugs were significantly different from that of the untreated control (Table 1, legend). (A, B) Inhibitors of various protein kinases. Treatment with all drugs gives curves below that of the untreated control; this is consistent with loss of GFP-pol from the template, and an increase in the rapidly exchanging pool of the enzyme. Alster: alsterpaullone. (C, D) Other inhibitors. Curves for untreated controls are included in each case. Curves for actinomycin D (actD), and two histone deacetylase inhibitors (TSA, sodium butyrate) lie above that of the untreated control, indicating the drugs increase the fraction of GFP-pol bound to the template. In contrast, curves for the two proteasome inhibitors (lactacystin, MG132), and cisplatin all lie below, indicating they decrease the bound fraction.

transcriptional initiation (as a result of the activation of various signalling pathways [45–48]).

Proteasome inhibitors

Two proteasome inhibitors – MG132 and lactacystin – have little effect on the relative abundance of most mature transcripts [37,38], and so would be expected to have little effect on polymerase kinetics. However, both reduce the fraction of engaged polymerase (Fig. 2D).

Histone deacetylase inhibitors

Two histone deacetylase (HDAC) inhibitors – trichostatin (TSA) and sodium butyrate – are general activators of transcription, although they also have little effect on the relative abundance of most mature transcripts [41,42]. Consistent with their role as activators, they slightly increase the engaged fraction (Figs. 2C, D), although the curves were not as significantly different from the control as those of the other inhibitors (Table 1, legend).

Ultra-violet light

Irradiation with ultra-violet light (UV-C) induces damage in DNA that stalls RNA polymerase II like cisplatin; this also triggers the transcription-coupled repair pathway and eventually proteolytic degradation of the polymerase [33,36,48,49]. In contrast to the effects seen with cisplatin, irradiation increases the fraction of engaged GFP-pol in a dose-dependent manner to a maximum (Fig. 3). [The dose of 10 J/m^2 that gives this maximum induces one (endonuclease-sensitive) lesion every ~10 kbp [50]; it also reduces transcription to 22% of controls and transcription falls even further on continued growth in the absence of the drug (Table 1).]

Effect on transcription

The extent of transcriptional inhibition by the various drugs was determined by pulse labelling with BrU. Cells were grown without and with a drug for 1 or 2 h – the minimum and maximum exposure used for FLIP – and then for another 15 min in BrU; any RNA made during this short BrU pulse then becomes tagged with bromine, and was indirectly immunolabelled with Cy3. RNA polymerase II is responsible for ~65% of all transcription in the cell, and ~93% of all nucleoplasmic transcription [51]; therefore, nucleoplasmic Cy3 fluorescence

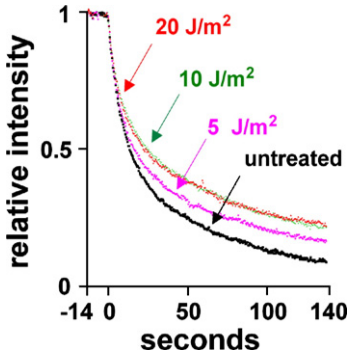

Fig. 3 – GFP-pol kinetics (analyzed using FLIP as in Fig. 1) in cells irradiated with different doses of UV-C. The laser used during FLIP had a power slightly greater than that used in Fig. 2 (it was newer), so the relative intensity given by untreated controls falls more rapidly and further. 5 and 10 J/m^2 progressively increase the fraction of engaged GFP-pol, but 20 J/m^2 gives no further effect. All curves were significantly different from all others, except for those given by 10 and 20 J/m^2 (i.e., $p<0.005$; significance assessed using relative intensities at 130 s, as in Table 1).

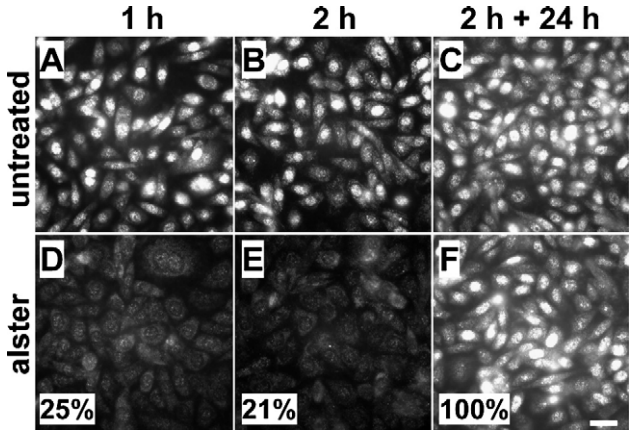

Fig. 4 – The effects of alsterpaullone on transcription. Cells were grown ± 50 μM alsterpaullone (alster) for 1 or 2 h, or for 2 h before cells were washed and regrown for 24 h in the absence of the drug (2 h + 24 h). 2.5 mM BrU was now added to the medium, and cells grown for a further 15 min; after fixation, the resulting Br-RNA was indirectly immunolabelled with Cy3, DNA counterstained with Hoechst 33342, and images of ~ 50 cells collected using a CCD camera. Six typical fields illustrating Cy3 fluorescence (marking newly made Br-RNA) are shown. Bar: 30 μm. (A–C) After growth in the absence of drug, Cy3 fluorescence per cell (which reflects the transcription rate) remains constant but the total amount of fluorescence in the field increases (as cell number increases). (D–F) After growth for 1 or 2 h in the drug, nuclei are less intensely labelled as the drug inhibits transcription, but Cy3 fluorescence recovers almost to levels found in untreated controls after the 24-h chase. The intensity of nucleoplasmic Cy3 signal (expressed as a percentage of the untreated counterparts shown above) is indicated.

reflects polymerase II activity. Fig. 4 illustrates a typical experiment. Growth in alsterpaullone (alster) for 1 and 2 h reduces Cy3 signal to 25% and 21% of the controls (compare Figs. 4A, B with Figs. 4D, E). If the treated cells are now washed and regrown in the absence of the drug, the signal recovers to the level found in controls (compare Fig. 4C with Fig. 4F). We conclude that alsterpaullone reversibly inhibits RNA polymerase II activity.

Results for the other drugs are illustrated in Table 1; all inhibit transcription to some extent. This inhibition could generally be reversed by continued growth in the absence of drug; however, there was only partial recovery after treatment with KM05283, actinomycin D, and TSA – and no recovery after UV-irradiation (Table 1, legend). Clearly, the exposures used are usually too small to irreversibly inhibit transcription.

Discussion

RNA polymerase II is the enzyme responsible for transcribing most eukaryotic genes, and exhaustive studies have given us detailed information on how it works *in vitro*; here, we assess how various transcriptional inhibitors affect its dynamics *in vivo*. We had previously established a stable cell line that expresses the largest (catalytic) subunit of the polymerase tagged with GFP, and had shown that this tagged polymerase is fully functional as it is required to sustain life at 39 °C [2]. Photobleaching studies also showed that this line contains two major populations of tagged polymerase – one large and rapidly diffusing, and the second smaller and relatively immobile; the former probably represents the inactive pool, and the latter the active polymerases engaged on their templates [5,6]. Inhibitors were used at concentrations sufficient to suppress transcription significantly but reversibly (Table 1), and they acted in two general ways: one class decreased the engaged fraction, the other increased it (Figs. 2 and 3). As with all discussions of inhibitors, this one comes with the caveat that the inhibitors may act in ways other than the intended one.

Transcriptional initiation and elongation depend on CTD phosphorylation (Introduction), so we would expect kinase inhibitors to prevent the polymerase from engaging, and therefore to increase the size of the rapidly exchanging pool; this is the case (Figs. 2A, B). Indeed, inspection of Table 1 shows there is a good correlation between the degree of transcriptional inhibition and the fraction of engaged polymerase (as assessed by FLIP). [In Table 1, kinase inhibitors are listed in the order that reflects the percentage of engaged polymerase.] For example, H-8 is the weakest transcriptional inhibitor (at the concentration and exposure used here) and incubation in the drug for 1 and 2 h reduces the incorporation of BrU to 81% and 73% of that of untreated controls; levels of engaged polymerase are reduced to roughly the same extent (i.e., to 69%). At the other extreme, the strongest inhibitor (alsterpaullone) under our conditions reduces incorporation to 25% and 21%, and leaves only 17% polymerase on the template. Each of these different inhibitors inhibit different targets to different degrees, but they all inhibit phosphorylation of Ser2 in the CTD by P-TEFb (Introduction); therefore, P-TEFb may well be the common target. Heat shock – another effective transcriptional inhibitor – acts like these kinase inhibitors to reduce the engaged fraction [6].

Agents that slow or stall elongating/terminating polymerases, or facilitate polymerase initiation, might be expected to increase the engaged fraction. Actinomycin D, irradiation with UV-C, and the HDAC inhibitors (TSA, butyrate) all seem to act in this general way (Figs. 2C and 3), one by creating a temporary obstacle (through intercalator binding), the second a more permanent one (through the photo-generation of a permanently linked adduct), and the third by making it easier for the enzyme to load on to its nucleosome-covered template. In contrast, cisplatin – which stalls the polymerase *in vitro* like UV light – has the opposite effect; it reduces the engaged fraction (Fig. 2C). This is consistent with stalling activating transcription-coupled repair followed by the proteolytic removal of the stalled enzyme from the template, and/or a reduction in initiation [45–48,52]. As many of the adducts generated by UV-irradiation and cisplatin are repaired by the same pathway [31,32,53], why does one increase the fraction of engaged polymerase while the other reduces it? We can speculate the differences might arise from the different kinds of damage the two induce, which signalling pathways are activated, and how rapidly the resulting signals feed back to

the polymerase. [Neither actinomycin D, UV-C, nor cisplatin induced substantial degradation of GFP-pol, as treatment induced only marginal falls in total signals of 15%, 10%, and 6%, respectively (data not shown). However, it remains possible that changes in the smaller bound fraction were masked by the changes in the larger soluble pool.]

Two proteasome inhibitors – MG132 and lactacystin – reduce the engaged fraction. Taken at face value, this is consistent with efficient transcription normally depending on continued proteolysis; we have no obvious explanation of why this might be so. We know that stalled polymerases are removed by the proteosome (above), so we might expect these two inhibitors to increase the engaged fraction. We might also expect this effect to be substantial if significant numbers of polymerases stall under normal circumstances, as is the case in the fly [54]. As we find the opposite effect, it may be that stalling generates a signal that slows engagement of new polymerases, and/or enhances disengagement of active ones. But as stated above, these inhibitors may have other, unexpected, effects – and the GFP tag may even play unexpected roles. For example, it has recently been found that EGFP can inhibit polyubiquitinylation [55]. In the face of such conflicting results, and in the absence of more data, we prefer not to speculate further.

Taken together, these results illustrate how complicated the response to transcriptional inhibition is. They also reinforce the emerging view that the transcription complex is both an important sensor and effector at the heart of a complex signalling network [48,52,53]. As a number of these inhibitors are being used therapeutically, it is clearly important to establish what effects they might have on this complex circuitry.

Acknowledgments

We thank the Biotechnology and Biological Sciences Research Council for support, Colin Blackmore for help with image analysis using 'Definiens Developer' software, and Nigel Burroughs and Howsun Jow for help in analyzing the kinetics.

REFERENCES

[1] H. Tsuji, Y. Matsudo, S. Tsuji, F. Hanaoka, M. Hyodo, T. Hori, Isolation of temperature-sensitive CHO-K1 cell mutants exhibiting chromosomal instability and reduced DNA synthesis at nonpermissive temperature, Somat. Cell Mol. Genet. 16 (1990) 461–476.

[2] K. Sugaya, S. Sasanuma, J. Nohata, T. Kimura, E. Hongo, T. Higashi, M. Morimyo, H. Tsuji, K. Mita, Cloning and sequencing for the largest subunit of Chinese hamster RNA polymerase II gene: identification of a mutation related to abnormal induction of sister chromatid exchanges, Gene 194 (1997) 267–272.

[3] K. Sugaya, M. Vigneron, P.R. Cook, Mammalian cell lines expressing functional RNA polymerase II tagged with the green fluorescent protein, J. Cell Sci. 113 (2000) 2679–2683.

[4] K. Sugaya, S. Sasanuma, P.R. Cook, K. Mita, A mutation in the largest (catalytic) subunit of RNA polymerase II and its relation to the arrest of the cell cycle in G(1) phase, Gene 274 (2001) 77–81.

[5] H. Kimura, K. Sugaya, P.R. Cook, The transcription cycle of RNA polymerase II in living cells, J. Cell Biol. 159 (2002) 777–782.

[6] M. Hieda, H. Winstanley, P. Maini, F.J. Iborra, P.R. Cook, Different populations of RNA polymerase II in living mammalian cells, Chromosome Res. 13 (2005) 135–144.

[7] M. Becker, C. Baumann, S. John, D.A. Walker, M. Vigneron, J.G. McNally, G.L. Hager, Dynamic behavior of transcription factors on a natural promoter in living cells, EMBO Rep. 3 (2002) 1188–1194.

[8] S.M. Janicki, T. Tsukamoto, S.E. Salghetti, W.P. Tansey, K.V. R. Sachidanandam, T. Prasanth, Y. Ried, E. Shav-Tal, R.H. Bertrand, D.L. Singer, From silencing to gene expression: real-time analysis in single cells, Cell 116 (2004) 683–698.

[9] J.R. Chubb, T. Trcek, S.M. Shenoy, R.H. Singer, Transcriptional pulsing of a developmental gene, Curr. Biol. 16 (2006) 1018–1025.

[10] X. Darzacq, R.H. Singer, Y. Shav-Tal, Dynamics of transcription and mRNA export, Curr. Opin. Cell Biol. 17 (2005) 332–339.

[11] G.L. Hager, C. Elbi, T.A. Johnson, T. Voss, A.K. Nagaich, R.L. Schiltz, Y. Qiu, S. John, Chromatin dynamics and the evolution of alternate promoter states, Chromosome Res. 14 (2006) 107–116.

[12] A. Meinhart, T. Kamenski, S. Hoeppner, S. Baumli, P. Cramer, A structural perspective of CTD function, Genes Dev. 19 (2005) 1401–1415.

[13] S. Trigon, H. Serizawa, J.W. Conaway, R.C. Conaway, S.P. Jackson, M. Morange, Characterization of the residues phosphorylated in vitro by different C-terminal domain kinases, J. Biol. Chem. 273 (1998) 6769–6775.

[14] C.K. Ho, S. Shuman, Distinct roles for CTD Ser-2 and Ser-5 phosphorylation in the recruitment and allosteric activation of mammalian mRNA capping enzyme, Mol. Cell 3 (1999) 405–411.

[15] B.M. Peterlin, D.H. Price, Controlling the elongation phase of transcription with P-TEFb, Mol. Cell 23 (2006) 297–305.

[16] Y. Hirose, J.L. Manley, RNA polymerase II is an essential mRNA polyadenylation factor, Nature 395 (1998) 93–96.

[17] Y. Hirose, R. Tacke, J.L. Manley, Phosphorylated RNA polymerase II stimulates pre-mRNA splicing, Genes Dev. 13 (1999) 1234–1239.

[18] Y.W. Fong, Q. Zhou, Stimulatory effect of splicing factors on transcriptional elongation, Nature 414 (2001) 929–933.

[19] D.A. Zorio, D.L. Bentley, The link between mRNA processing and transcription: communication works both ways, Exp. Cell Res. 296 (2004) 91–97.

[20] L.A. Chodosh, A. Fire, M. Samuels, P.A. Sharp, 5,6-Dichloro-1-beta-D-ribofuranosylbenzimidazole inhibits transcription elongation by RNA polymerase II in vitro, J. Biol. Chem. 264 (1989) 2250–2257.

[21] S.P. Davies, H. Reddy, M. Caivano, P. Cohen, Specificity and mechanism of action of some commonly used protein kinase inhibitors, Biochem. J. 351 (2000) 95–105.

[22] J. Bain, H. McLauchlan, M. Elliott, P. Cohen, The specificities of protein kinase inhibitors: an update, Biochem. J. 371 (2003) 199–204.

[23] M.E. Noble, J.A. Endicott, L.N. Johnson, Protein kinase inhibitors: insights into drug design from structure, Science 303 (2004) 1800–1805.

[24] M.F. Dubois, V.T. Nguyen, S. Bellier, O. Bensaude, Inhibitors of transcription such as 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole and isoquinoline sulfonamide derivatives (H-8 and H-7) promote dephosphorylation of the carboxyl-terminal domain of RNA polymerase II largest subunit, J. Biol. Chem. 269 (1994) 13331–13336.

[25] J. Medlin, P. Uguen, A. Taylor, D.L. Bentley, S. Murphy, The C-terminal domain of pol II and a DRB-sensitive kinase are

required for 3′ processing of U2 snRNA, EMBO J. 22 (2003) 925–934.
[26] J. Medlin, A. Scurry, A. Taylor, F. Zhang, B.M. Peterlin, S. Murphy, P-TEFb is not an essential elongation factor for the intronless human *U2* snRNA and histone *H2b* genes, EMBO J. 24 (2005) 4154–4165.
[27] G. Bird, D.A. Zorio, D.L. Bentley, RNA polymerase II carboxy-terminal domain phosphorylation is required for cotranscriptional pre-mRNA splicing and 3′-end formation, Mol. Cell Biol. 24 (2004) 8699–8963.
[28] M. Ljungman, M.T. Paulsen, The cyclin-dependent kinase inhibitor roscovitine inhibits RNA synthesis and triggers nuclear accumulation of p53 that is unmodified at Ser15 and Lys382, Mol. Pharmacol. 60 (2001) 785–789.
[29] L.T. Lam, O.K. Pickeral, A.C. Peng, A. Rosenwald, E.M. Hurt, J.M. Giltnane, L.M. Averett, H. Zhao, R.E. Davis, M. Sathyamoorthy, L.M. Wahl, E.D. Harris, J.A. Mikovits, A.P. Monks, M.G. Hollingshead, E.A. Sausville, L.M. Staudt, Genomic-scale measurement of mRNA turnover and the mechanisms of action of the anti-cancer drug flavopiridol, Genome Biol. 2 (2001) (RESEARCH0041).
[30] I.A. Vlasova, J. McNabb, A. Raghavan, C. Reilly, D.A. Williams, K.A. Bohjanen, P.R. Bohjanen, Coordinate stabilization of growth-regulatory transcripts in T cell malignancies, Genomics 86 (2005) 159–171.
[31] Z.H. Siddik, Cisplatin: mode of cytotoxic action and molecular basis of resistance, Oncogene 22 (2003) 7265–7279.
[32] S. Tornaletti, S.M. Patrick, J.J. Turchi, P.C. Hanawalt, Behavior of T7 RNA polymerase and mammalian RNA polymerase II at site-specific cisplatin adducts in the template DNA, J. Biol. Chem. 278 (2003) 35791–35797.
[33] D.B. Bregman, R. Halaban, A.J. van Gool, K.A. Henning, E.C. Friedberg, S.L. Warren, UV-induced ubiquitination of RNA polymerase II: a novel modification deficient in Cockayne syndrome cells, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 11586–11590.
[34] M.A. Whiteside, D.T. Chen, R.A. Desmond, S.A. Abdulkadir, G.L. Johanning, A novel time-course cDNA microarray analysis method identifies genes associated with the development of cisplatin resistance, Oncogene 23 (2004) 744–752.
[35] G. Nalepa, M. Rolfe, J.W. Harper, Drug discovery in the ubiquitin–proteasome system, Nat. Rev., Drug Discov. 5 (2006) 596–613.
[36] J.N. Ratner, B. Balasubramanian, J. Corden, S.L. Warren, D.B. Bregman, Ultraviolet radiation-induced ubiquitination and proteasomal degradation of the large subunit of RNA polymerase II. Implications for transcription-coupled DNA repair, J. Biol. Chem. 273 (1998) 5184–5189.
[37] J. Zimmermann, D. Erdmann, I. Lalande, R. Grossenbacher, M. Noorani, P. Furst, Proteasome inhibitor induced gene expression profiles reveal overexpression of transcriptional regulators ATF3, GADD153 and MAD1, Oncogene 19 (2000) 2913–2920.
[38] E.H. Yew, N.S. Cheung, M.S. Choy, R.Z. Qi, A.Y. Lee, Z.F. Peng, A.J. Melendez, J. Manikandan, E.S. Koay, L.L. Chiu, W.L. Ng, M. Whiteman, J. Kandiah, B. Halliwell, Proteasome inhibition by lactacystin in primary neuronal cells induces both potentially neuroprotective and pro-apoptotic transcriptional responses: a microarray analysis, J. Neurochem. 94 (2005) 943–956.
[39] J.R. Davie, Inhibition of histone deacetylase activity by butyrate, J. Nutr. 133 (2003) 2485S–2493S.
[40] C. Monneret, Histone deacetylase inhibitors, Eur. J. Med. Chem. 40 (2005) 1–13.
[41] T. Chiba, O. Yokosuka, K. Fukai, H. Kojima, M. Tada, M. Arai, F. Imazeki, H. Saisho, Cell growth inhibition and gene expression induced by the histone deacetylase inhibitor, trichostatin A, on human hepatoma cells, Oncology 66 (2004) 481–491.
[42] Y. Tabuchi, I. Takasaki, T. Doi, Y. Ishii, H. Sakai, T. Kondo, Genetic networks responsive to sodium butyrate in colonic epithelial cells, FEBS Lett. 580 (2006) 3035–3041.
[43] H. Kimura, M. Hieda, P.R. Cook, Measuring histone and polymerase dynamics in living cells, Methods Enzymol. 375 (2004) 381–393.
[44] F.J. Iborra, D.A. Jackson, P.R. Cook, The path of transcripts from extra-nucleolar synthetic sites to nuclear pores: transcripts in transit are concentrated in discrete structures containing SR proteins, J. Cell Sci. 111 (1998) 2269–2282.
[45] T. Furuta, T. Ueda, G. Aune, A. Sarasin, K.H. Kraemer, Y. Pommier, Transcription-coupled nucleotide excision repair as a determinant of cisplatin sensitivity of human cells, Cancer Res. 62 (2002) 4899–4902.
[46] A. Tremeau-Bravard, T. Riedl, J.M. Egly, M.E. Dahmus, Fate of RNA polymerase II stalled at a cisplatin lesion, J. Biol. Chem. 279 (2004) 7751–7759.
[47] Y. Jung, S.J. Lippard, RNA polymerase II blockage by cisplatin-damaged DNA. Stability and polyubiquitylation of stalled polymerase, J. Biol. Chem. 281 (2006) 1361–1370.
[48] J.P. Laine, J.M. Egly, When transcription and repair meet: a complex system, Trends Genet. 22 (2006) 430–436.
[49] Z. Luo, J. Zheng, Y. Lu, D.B. Bregman, Ultraviolet radiation alters the phosphorylation of RNA polymerase II large subunit and accelerates its proteasome-dependent degradation, Mutat. Res. 486 (2001) 259–274.
[50] J.I. Williams, J.E. Cleaver, Removal of T4 endonuclease V-sensitive sites from SV40 DNA after exposure to ultraviolet light, Biochim. Biophys. Acta 562 (1979) 429–437.
[51] A. Pombo, D.A. Jackson, M. Hollinshead, Z. Wang, R.G. Roeder, P.R. Cook, Regional specialization in human nuclei: visualization of discrete sites of transcription by RNA polymerase III, EMBO J. 18 (1999) 2241–2253.
[52] J.Q. Svejstrup, Rescue of arrested RNA polymerase II complexes, J. Cell Sci. 116 (2003) 447–451.
[53] T.T. Saxowsky, P.W. Doetsch, RNA polymerase encounters with DNA damage: transcription-coupled repair or transcriptional mutagenesis? Chem. Rev. 106 (2006) 474–488.
[54] A. Law, K. Hirayoshi, T. O'Brien, J.T. Lis, Direct cloning of DNA that interacts in vivo with a specific protein: application to RNA polymerase II and sites of pausing in *Drosophila*, Nucleic Acids Res. 26 (1998) 919–924.
[55] M. Baens, H. Noels, V. Broeckx, S. Hagens, S. Fevery, A.D. Billiau, H. Vankelecom, P. Marynen, P., The dark side of EGFP: defective polyubiquitination, PLoS ONE 1 (2006) e54.