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Cotranscriptional coupling of splicing factor recruitment and precursor messenger RNA splicing in mammalian cells

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Coupling between transcription and RNA processing is a key gene regulatory mechanism. Here we use chromatin immunoprecipitation to detect transcription-dependent accumulation of the precursor mRNA (pre-mRNA) splicing factors hnRNP A1, U2AF65 and U1 and U5 snRNPs on the intron-containing human *FOS* gene. These factors were poorly detected on intronless heat-shock and histone genes, a result that opposes direct recruitment by RNA polymerase II (Pol II) or the cap-binding complex *in vivo*. However, an observed RNA-dependent interaction between U2AF65 and active forms of Pol II may stabilize U2AF65 binding to intron-containing nascent RNA. We establish chromatin-RNA immunoprecipitation and show that *FOS* pre-mRNA is cotranscriptionally spliced. Notably, the topoisomerase I inhibitor camptothecin, which stalls elongating Pol II, increased cotranscriptional splicing factor accumulation and splicing in parallel. This provides direct evidence for a kinetic link between transcription, splicing factor recruitment and splicing catalysis.

Many pre-mRNA processing events have been shown to occur during gene transcription, and it is believed that the coupling of transcription to specific mRNA maturation steps is a key regulatory mechanism[1–4]. A clear illustration of this concept is the capping of RNA at the 5′ end. The capping enzymes bind the C-terminal domain (CTD) of the large subunit of Pol II, ensuring that Pol II transcripts receive the 7-methylguanosine cap, which is important for many aspects of mRNA stability, further processing and translation, as well as for nuclear export of small nuclear RNA (snRNA)[5]. The capping enzymes are concentrated in upstream regions of active genes *in vivo*, consistent with their action during early stages of transcription[6–8]. Thus, the mechanistic coupling of capping to transcription is crucial for the expression of Pol II genes.

The observation that pre-mRNA splicing can occur cotranscriptionally (that is, while the RNA is still attached to the DNA by Pol II) indicates that splicing and transcription are at least temporally and perhaps mechanistically coupled[2,9]. Pre-mRNA splicing, an essential step in the expression of intron-containing genes, requires the assembly and activity of a large, multicomponent complex called the spliceosome[10]. Reconstitution of pre-mRNA splicing *in vitro* indicates that formation of the active spliceosome depends on the association of the spliceosomal small nuclear ribonucleoproteins (snRNPs U1 and U2, and the U4-U6-U5 tri-snRNP) and numerous non-snRNP splicing factors with the pre-mRNA. Although the two-step *trans*-esterification reaction that cleaves introns from exons and ligates two exons together is carried out within the active spliceosome, it is currently thought that selection of intron and exon boundaries reflects an earlier step, in which the 5′ and 3′ splice sites are recognized by base-pairing interactions between the pre-mRNA and the U1 and U2 snRNAs, respectively[11]. This early step can be influenced by the activities of non-snRNP splicing factors that enhance or suppress interaction of these snRNPs with 5′ and 3′ splice sites. An example is the factor U2AF65, which binds the polypyrimidine tract adjacent to 3′ splice sites and enhances U2 snRNP recruitment to the branchpoint. Regulation of splice-site selection in higher eukaryotes results in alternative splicing, currently thought to occur in 50%–70% of human genes, and thereby generates multiple protein products from single genes[11].

To what extent is splicing cotranscriptional, and how are splicing factors recruited to active genes? Until recently, opportunities to directly examine cotranscriptional pre-mRNA splicing events were extremely limited, but the application of chromatin immunoprecipitation (ChIP; see **Fig. 1**) has now enabled the detection of splicing factor accumulation on active genes of the yeast *Saccharomyces cerevisiae*[12–15]. These studies establish that, in yeast, spliceosomal snRNPs accumulate at positions along intron-containing genes that coincide with the synthesis of cognate splicing signals in nascent RNA.

There are several reasons to investigate the above questions in higher metazoans. First, their gene architecture is dramatically different; only 5% of yeast genes have introns, whereas the majority of human genes contain multiple introns and undergo alternative splicing. Second, many metazoan splicing regulators are absent in

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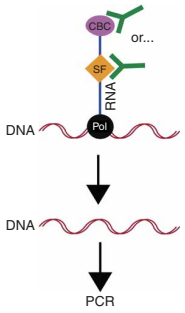

Figure 1 Schematic of the experimental approach, illustrating the expectation that ChIP with antibodies against CBC or potential splicing factors (SF) will pull down the chromatin regions to which proteins are attached through the nascent RNA (blue line) and Pol II.

yeast. Third, key differences in transcriptional machinery make it likely that additional coupling mechanisms apply in metazoans[4]. For example, the number of heptad repeats in the Pol II CTD as well as its sequence variability are much greater in human than yeast. In yeast, the CTD is not required for pre-mRNA splicing, whereas it is required in human cells[16,17]. Therefore, we transferred the ChIP approach to human tissue-culture cells to address *in vivo* splicing mechanisms. Here we analyze the accumulation of the cap-binding complex (CBC), heterogeneous nuclear ribonucleoprotein (hnRNP) A1, U1 and U5 snRNPs and U2AF65 on paused and induced human genes, with and without introns. Moreover, we show that the topoisomerase I inhibitor camptothecin, which interferes with Pol II elongation, leads to enhanced cotranscriptional accumulation of these splicing factors on the *FOS* gene encoding the FOS protein. Finally, we establish a new assay, chromatin-RNA immunoprecipitation (ChRIP), through which we show that *FOS* pre-mRNA splicing is cotranscriptional and strongly increased by camptothecin treatment. The ChRIP assay should enable future analysis of any cotranscriptional RNA processing event of interest.

RESULTS

Accumulation of CBC and hnRNP A1 on induced genes

Our aim was to use ChIP to detect the recruitment of RNA-binding proteins to endogenous mammalian transcription units (**Fig. 1**). A prerequisite is that nascent transcription complexes be detectable in downstream gene regions. Therefore, the distribution of Pol II along two inducible genes of similar length—*FOS*, which contains three introns, and *HSPA1B*, which is intronless and encodes the HSP70 heat-shock protein—was investigated in human A431 cells[18,19]. When uninduced, transcriptional pausing is known to occur in both genes after 20–40 nucleotides (nt)[20–22]. ChIPs with antibodies specific for Pol II confirmed that in uninduced cells, Pol II was exclusively detected in promoter-proximal gene regions (**Fig. 2**). Upon induction of *FOS* with calcium ionophore or of *HSPA1B* with sodium arsenite, Pol II became detectable in downstream regions of both genes. The Pol II antibody specific for a non-CTD epitope was more effective in recovering downstream regions than the antibody against Pol IIa (the hypophosphorylated CTD form), probably owing to phosphorylation of the CTD during initiation and elongation (**Fig. 2a**,**b**,**e**,**f**). Consistent with this interpretation, phosphorylated Pol II CTD epitopes were detected downstream in both induced genes (data not shown). These data demonstrate the robustness of both transcriptional induction protocols and show that elongating transcription complexes are detectable in downstream regions of induced *FOS* and *HSPA1B*.

To begin to investigate cotranscriptional accumulation of RNA-binding proteins, antibodies specific for the CBP80 subunit of the cap-binding complex (CBC) were used for ChIP. We anticipated that the CBC should bind the 5′ end of every capped Pol II transcript (**Fig. 1**). Capping occurs after only 20–30 nt of transcription for paused heat-shock genes in *Drosophila melanogaster*[21], raising the possibility that CBC might be among the earliest factors to bind nascent RNA. Consistent with the pausing data, we found that in uninduced cells, CBC was robustly detectable at the promoters of both genes and undetectable in downstream regions (**Fig. 2c**,**g**). This indicates that CBC is cotranscriptionally recruited to very short nascent RNAs. Upon gene induction, CBC became detectable in downstream *FOS* and *HSPA1B* gene regions (**Fig. 2c**,**g**), confirming the presence of nascent RNA and the efficacy of the assay.

This analysis was extended to the hnRNP A1 protein, an abundant nuclear protein with roles in transcription, pre-mRNA splicing and nuclear export[23]. hnRNP A1 was robustly detectable on *FOS* in induced but not uninduced cells (**Fig. 2d**), indicating that hnRNP A1 association with *FOS* depends on transcription beyond the pause site. The observation that hnRNP A1 was poorly detectable on *HSPA1B* with or without transcriptional activity (**Fig. 2h**) further suggests that hnRNP A1 associates with *FOS* but not *HSPA1B* nascent RNA. Although hnRNP A1–binding sites do not conform to a strict consensus[24–26], scanning of the *FOS* pre-mRNA sequence revealed at least four potential hnRNP A1–binding sites (UAGNNNUAG or UAGGGA) in the body of the transcript, with the first match occurring in intron 1 at 374–386 nt. *HSPA1B* mRNA did not contain any putative hnRNP A1–binding sites, suggesting that the detection of hnRNP A1 on *FOS* reflects direct binding to *FOS* nascent RNA.

Camptothecin increases splicing factor abundance on *FOS*

To investigate the accumulation of core components of the pre-mRNA splicing machinery at sites of transcription, antibodies specific for U2AF65, for the U1-70K component of the U1 snRNP and for the 116K component of the U5 snRNP were used in ChIPs of A431 cells induced or uninduced for *FOS* transcription. None of these factors was detectable on *FOS* in uninduced cells (**Fig. 3a–d**). Because CBC and Pol II are abundant in the promoter-proximal region under these conditions (see above), this suggests that U1 and U5 snRNPs and U2AF65 do not detectably associate with the paused polymerase, the CBC or the short nascent transcript. However, upon transcriptional induction, all three factors were detectable within the transcription unit (see **Fig. 3** for *P*-values), with the exception that U2AF65 was not detectable in the promoter-proximal region corresponding to exon 1. Comparing the induced to the uninduced signals, U1-70K, U2AF65 and U5-116K are, respectively, about two-, three- and seven-fold enriched at their peaks upon induction. As a first test for whether these splicing factors might be recruited to intronless genes, their abundances were examined on the constitutively active, intronless histone *HIST1H2AB* gene; relative to the uninduced controls, these factors were only poorly detectable on *HIST1H2AB*, although Pol II and CBC were well detected (**Fig. 3e–h** and data not shown). We conclude from this that U1 and U5 snRNPs as well as U2AF65 accumulate on intron-containing *FOS* in a transcription-dependent manner.

If splicing factor accumulation at any given gene position is influenced by the rate at which Pol II progresses through the gene, then perturbation of transcription elongation may influence the levels of splicing factor accumulation. The fast-acting drug camptothecin

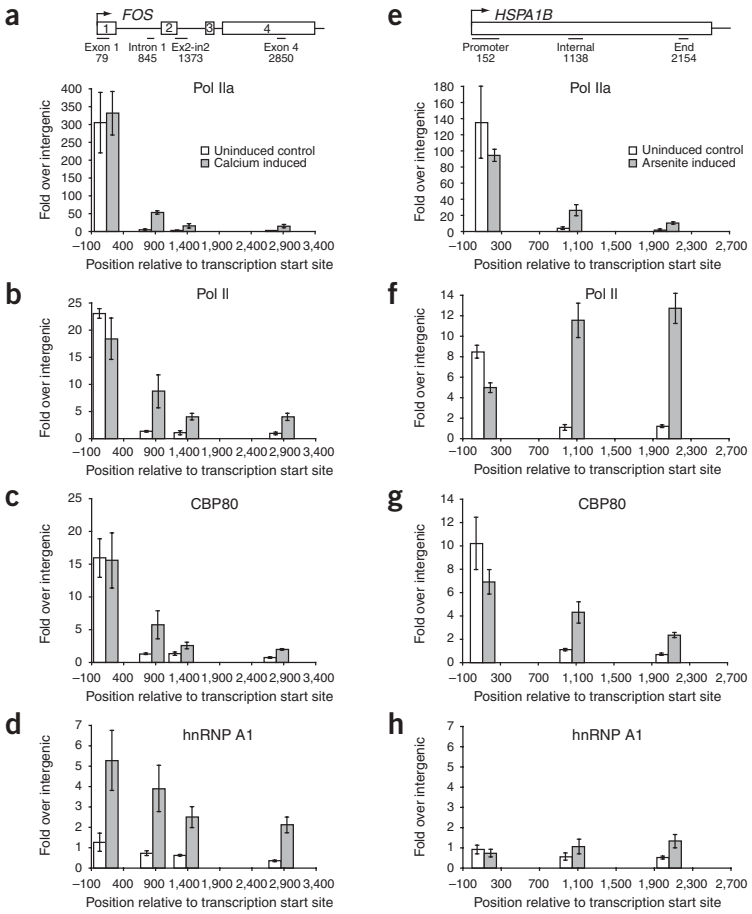

Figure 2 Induction of *FOS* and *HSPA1B* transcription leads to downstream accumulation of RNA Pol II, CBP80 and hnRNP A1. (**a**–**h**) ChIP results. Schematics of *FOS* and *HSPA1B* are shown, with black lines indicating gene regions amplified by primer sets, identified by the nucleotide at the center of the amplified region. *FOS* transcription was induced with calcium ionophore (gray); control cells were treated with DMSO alone (white). *HSPA1B* transcription was induced with sodium arsenite (gray); control cells were untreated (white). All values are relative to nonimmune background ChIP experiments and normalized to an intergenic control region, set as 1. Accumulation profiles of unphosphorylated Pol IIa (**a**,**e**) or total Pol II (**b**,**f**) on *FOS* and *HSPA1B* reveals paused Pol II at promoter regions on genes before induction and no accumulation in downstream regions. Upon gene induction, Pol II accumulates in downstream regions. CBP80 (**c**,**g**) accumulates solely at promoter regions when genes are uninduced and is detectable in downstream gene regions upon induction. HnRNP A1 (**d**,**h**) accumulates cotranscriptionally on the induced *FOS* gene but not significantly ($P > 0.05$) on induced *HSPA1B*. No hnRNP A1 accumulation was detected on uninduced genes. Each bar shows average and s.e.m. of at least three independent experiments. Number of experiments were as follows (listed as *n* uninduced, *n* induced): (**a**) 4, 4; (**b**) 3, 3; (**c**) 4, 3; (**d**) 4, 3. (**e**) 4, 4; (**f**) 4, 8; (**g**) 3, 5; (**h**) 3, 4.

inhibits elongation by blocking topoisomerase I–mediated relief of DNA supercoiling that occurs during transcription[27,28]. Over 30 sites of topoisomerase I activity are distributed throughout the *FOS* gene and have been mapped with camptothecin[19], raising the possibility that camptothecin may create physical blocks to Pol II movement without causing its release from the DNA template. Consistent with this, Pol II and CBC ChIPs of cells treated with a short pulse of camptothecin in combination with induction revealed levels characteristic of induction alone (**Fig. 3a** and data not shown). Notably, camptothecin treatment enhanced all splicing factor signals along *FOS* (**Fig. 3b–d**). Comparing the induced to the uninduced signals, U1-70K, U2AF65 and U5-116K are, respectively, about 4.5-, 6- and 12-fold enriched at their peaks in the presence of camptothecin. In contrast, camptothecin treatment did not induce association of splicing factors with the intronless *HIST1H2AB* gene, which is not a camptothecin target[29], providing an important specificity control (**Fig. 3f–h**). Therefore, we conclude that camptothecin treatment amplifies splicing factor signals by stalling nascent RNPs and giving the nascent RNA more time to bind splicing factors.

Splicing factors do not accumulate on intronless *HSPA1B*

As a robust test for splicing factor recruitment to an intronless gene, we took advantage of the intronless *HSPA1B* heat-shock gene, which is also a camptothecin target with multiple topoisomerase-sensitive sites distributed throughout the gene[28,30]. This gene showed a massive downstream concentration of Pol II upon induction with arsenite, whereas the U1 and U5 snRNPs were not detected at any position along induced *HSPA1B*, with or without camptothecin (**Fig. 4**). U2AF65 signals were similarly low; we note that signal above the intergenic control was nearly significant ($P < 0.08$) at the internal position, but this signal was not enhanced by camptothecin treatment. We verified by reverse-transcription (RT)-PCR that arsenite treatment did not impair splicing of *MYC* pre-mRNA (data not shown) and that all of the factors under study remain nuclear after arsenite treatment (**Supplementary Fig. 1** online); notably, a small proportion of the total hnRNP A1 signal was detectable in cytoplasmic stress granules after arsenite treatment. Thus, like the intronless histone *HIST1H2AB* transcription unit, *HSPA1B* accumulates splicing factors poorly.

Interactions between Pol II and splicing factors

It has previously been proposed that direct binding to Pol II leads to splicing factor recruitment to active transcription units[1,31]; indeed, snRNPs and U2AF65 have been shown to associate with Pol II in a variety of mammalian extracts[32–35]. Moreover, a recent study has shown that the CBC is required for cotranscriptional spliceosome assembly in yeast[13], raising the possibility that Pol II, CBC or both might recruit splicing factors. Therefore, we designed two experiments to test for interactions among splicing factors, Pol II and CBC. In the first approach, A431 cells were metabolically labeled with [^{32}P]orthophosphate, immunoprecipitated without prior cross-linking and analyzed by fluorography for the presence of specific snRNAs (**Fig. 5a**). As expected, anti–U1-70K pulled down only the U1 snRNA, and anti–U5-116K pulled down U4, U5 and U6 snRNAs. Anti-U2AF65 pulled down U2, U4, U5 and U6 snRNAs, consistent with the presence of U2AF65 in spliceosomes[10]. Quantification of multiple results

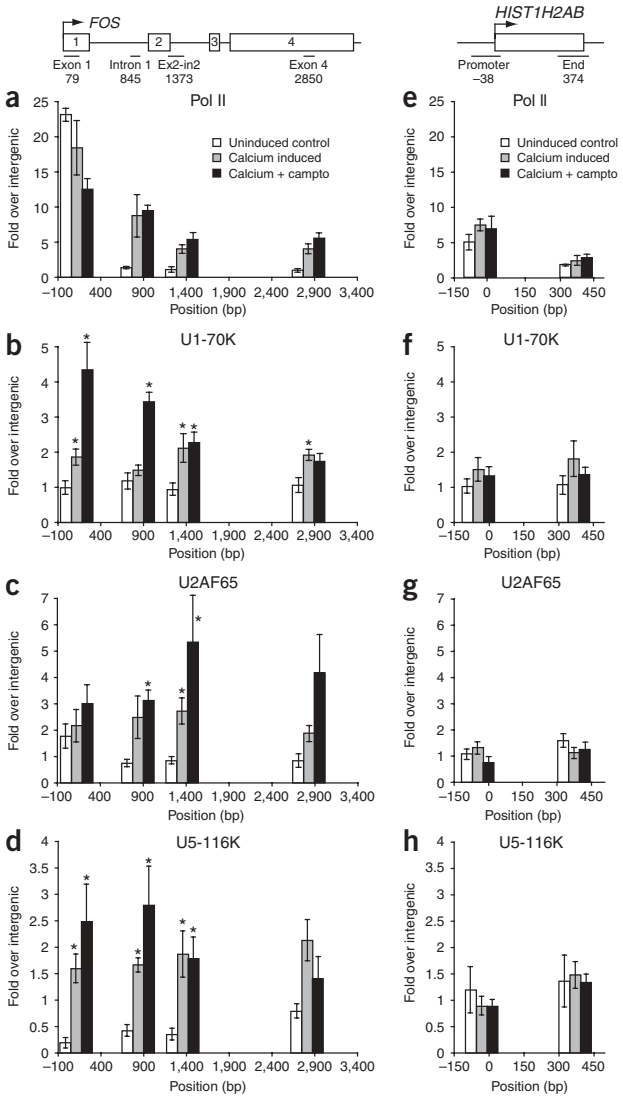

Figure 3 Splicing factors accumulate cotranscriptionally on the intron-containing *FOS* gene. (**a**–**h**) Distribution of Pol II (**a**,**e**), U1-70K (**b**,**f**), U2AF65 (**c**,**g**) and U5-116K (**d**,**h**) on *FOS* and *HIST1H2AB* genes. Positions are relative to transcription start (in base pairs). Schematics of *FOS* and histone *HIST1H2AB* (NCBI GeneID 8335) are shown as in **Figure 2**. A431 cells were treated with either DMSO (white), calcium ionophore (gray) or calcium ionophore and then camptothecin (black). Data are normalized and represented as in **Figure 2**. Asterisks indicate values significantly different from the uninduced values (Student's *t*-test $P \leq 0.05$). Numbers of experiments were as follows (listed as *n* with DMSO, *n* with calcium ionophore, *n* with calcium ionophore plus camptothecin): (**a**) 3, 4, 5; (**b**) 3, 4, 3; (**c**) 4, 7, 4; (**d**) 3, 4, 3; (**e**) 3, 4, 3; (**f**) 3, 4, 3; (**g**) 4, 3, 3; (**h**) 4, 4, 3.

indicated that antibodies specific for CBP80, the internal region of Pol II and the hypophosphorylated or hyperphosphorylated Pol II CTD did not pull down any snRNA above background (**Fig. 5a** and data not shown). By contrast, recovery of U1 and U2 snRNAs by Y12 (anti-Sm, core snRNP proteins) and anti–U1-70K, for example, gave signals 400- to 2,000-fold over background. Thus, interactions between snRNPs and Pol II or CBC were not detected.

In the second approach, anti-CBP80, anti–U1-70K, anti-U2AF65 and anti–U5-116K were used to immunoprecipitate unlabeled and un-cross-linked A431 cell extracts; phosphorylated forms of the Pol II large subunit in each immunoprecipitate were detected by western blotting using the H14 and H5 monoclonal antibodies against Pol II CTD repeats phosphorylated on Ser5 and Ser2, respectively. Neither form of Pol II was detected in CBP80, U1-70K or U5-116K immunoprecipitates (**Fig. 5b**). Given the limits of detection of the assay, we estimate that if Pol II does associate with any of these factors, it must be bound to $\leq$10% of each. Hypophosphorylated Pol II was also not detected, and identical results were obtained with hnRNP A1 (data not shown). Together with the results of the metabolic labeling experiment (see above), these data do not support a strong physical interaction between Pol II and the CBC, the U1 snRNP or the U5 snRNP. In contrast, hyperphosphorylated forms of Pol II did coimmunoprecipitate with U2AF65. Notably, RNase A treatment before immunoprecipitation nearly abolished detection of Pol II, suggesting that the association between active Pol II and U2AF65 is, at least in part, mediated by RNA.

Cotranscriptional splicing is enhanced by camptothecin

The observation that splicing factors, including the U5-116K protein component of the U4-U6-U5 tri-snRNP, accumulate on induced *FOS* suggests that splicing catalysis may also occur cotranscriptionally (**Fig. 3**). To address this, an experiment was designed in which active chromatin is immunopurified and the attached nascent RNA is amplified by RT-PCR (**Fig. 6a**); we named the assay chromatin-RNA immunoprecipitation (ChRIP). Antibodies specific for acetylated histone 4 (AcH4) were used to immunoprecipitate cross-linked extracts of A431 cells induced with calcium ionophore, with and without camptothecin. Acetylated histones were robustly detectable within *FOS* by ChIP under both conditions (**Supplementary Fig. 2** online). Extraction of RNA from anti-AcH4 immunoprecipitates followed by quantitative RT-PCR analysis indicated that both spliced and unspliced *FOS* RNA was associated with chromatin, with negligible contamination by poly(A)$^+$ mRNA (see **Supplementary Fig. 3** online for details). The ratio of signals for spliced over unspliced (pre-m)RNA for exon 1–exon 2 was $\sim$15 upon induction, whereas the analogous ratio for exon 3–exon 4 splicing was $\sim$45 (**Fig. 6b**,**c**). This indicates that *FOS* pre-mRNA undergoes splicing cotranscriptionally. Notably, camptothecin treatment led to a substantial increase in cotranscriptional splicing for both introns, yielding ratios of spliced to unspliced signals three- and two-fold higher, respectively, than with calcium induction alone (**Fig. 6b**,**c**). We conclude from this that cotranscriptional *FOS* splicing is promoted by obstruction of Pol II elongation with camptothecin, which also correlates with enhanced splicing factor accumulation at the gene (**Fig. 3**). These observations provide direct evidence that the kinetics of transcription by Pol II influences cotranscriptional spliceosome assembly and splicing.

DISCUSSION

Here we have shown that core components of the splicing machinery accumulate in a transcription-dependent manner on the *FOS* gene and that *FOS* pre-mRNA is cotranscriptionally spliced. This was made possible by ChIP with splicing factor–specific antibodies in human cells and by the development of a new assay, ChRIP, in which active chromatin is immunopurified and copurifying nascent RNA analyzed. The widespread belief that pre-mRNA splicing is cotranscriptional derives largely from pioneering work in *Chironomus tentans* and *Drosophila melanogaster*[36–40]. Because nascent RNA is generally such

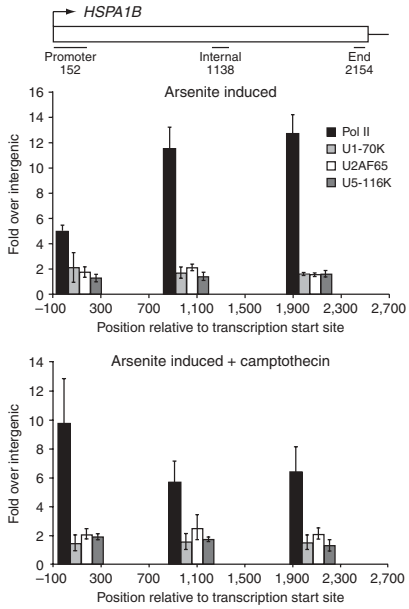

Figure 4 Splicing factors do not accumulate on the induced heat-shock gene, *HSPA1B*, in the presence or absence of camptothecin. Accumulation profile of total Pol II (black), U1-70K (light gray), U2AF65 (white) and U5-116K (dark gray) along induced *HSPA1B*. A431 cells were treated with sodium arsenite (upper chart) for 1 h, or for 45 min plus an additional 15 min with camptothecin (lower chart), before cross-linking and ChIP. Data are normalized and represented as in **Figure 2**. None of these values was significantly different from the intergenic control region (Student's *t*-test $P > 0.05$). Upper chart: Pol II, $n = 8$; U1-70K, $n = 3$; U2AF65, $n = 5$; U5-116K, $n = 3$. Lower chart: Pol II, $n = 3$; U1-70K, $n = 3$; U2AF65, $n = 3$; U5-116K, $n = 3$.

a small fraction of any given RNA species, it has been difficult to extend studies of cotranscriptional pre-mRNA splicing to mammalian cells. By considering splicing in the context of chromatin, one gains access to these rare RNA molecules and can address mechanistic questions such as the requirements for cotranscriptional splicing factor recruitment and splicing. Exemplifying this, our finding that the topoisomerase I inhibitor camptothecin increases splicing factor accumulation on *FOS* as well as cotranscriptional splicing levels provides direct evidence that cotranscriptional splicing events depend on the kinetics of RNA synthesis.

The position of splicing factor accumulation is meaningful, as it indicates the potentially important regions of the corresponding transcribed RNA. For example, CBC binds the 7-methylguanosine cap at the 5′ end of Pol II transcripts. Accordingly, CBC accumulation was high in promoter-proximal regions of transcriptionally paused *FOS* and *HSPA1B*, as well as in downstream regions of both induced genes. This is consistent with the notion that CBC binds very short nascent RNAs, within 40 nt of transcription for the paused genes. Because CBC has a role in cotranscriptional spliceosome assembly and splicing[13], these findings indicate that CBC is in a position to regulate cotranscriptional splicing at mammalian transcription units. Similarly, hnRNP A1 accumulation was detected in upstream regions of active *FOS*, consistent with the presence of a putative hnRNP A1 binding site early in intron 1. In contrast, U2AF65 was best detected in downstream regions of *FOS*, after transcription of the first 3′ splice site; thus, the U2AF65 distribution is consistent with its role in 3′ splice site definition.

Comparison of the mammalian and yeast ChIP data indicates several key differences in spliceosome assembly. First, the human U1 and U5 snRNPs were robustly detectable in upstream *FOS* regions, in exon 1 and intron 1. The presence of the U1 snRNP at this position, in the region of 5′ splice site synthesis, is consistent with the yeast data[12–15]. However, the detection of the U5 snRNP was surprising. Similar studies in yeast have revealed a clear separation in U1 and U5 snRNP accumulation, with the U5 snRNP appearing after 3′ splice site synthesis in all genes examined[13–15]. Because U5-116K protein did not coimmunoprecipitate with Pol II or associate with intronless genes, it seems unlikely that the U5 snRNP is brought to upstream regions of *FOS* by Pol II. These results are in agreement with yeast studies showing that U1 and U5 snRNPs are not recruited to intronless genes by Pol II or any other mechanism[12,13]. Instead, the mammalian data suggest either that the U5 snRNP makes early and relatively stable contacts with the 5′ splice site, as suggested by biochemical studies[41,42], or that the U5 snRNP is recruited to the gene in the context of a preassembled penta-snRNP complex[43,44].

Second, the yeast homolog of U2AF65, Mud2, accumulates on intronless as well as intron-containing genes[13]. In contrast, human U2AF65 was poorly detectable on the intronless histone and heat-shock genes *HIST1H2AB* and *HSPA1B*. *In vitro* experiments suggest that U2AF65 binds directly to Pol II during the transition from

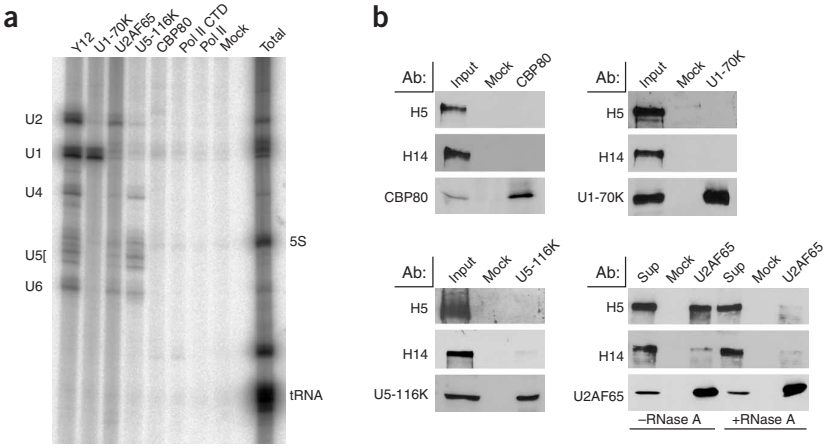

Figure 5 Coimmunoprecipitation of splicing factors with RNA Pol II without cross-linking. (**a**) Immunoprecipitation of ^{32}P-labeled snRNAs from A431 cells with Y12 (positive control) and antibodies to factors indicated above gel lanes. (**b**) Cell extracts were immunoprecipitated as in **a** with antibodies indicated above gel lanes. Precipitate and 0.3% of input material were western blotted with antibodies H5 and H14 to detect Ser2- and Ser5-phosphorylated Pol II CTD, respectively (indicated to left of each gel). In the U2AF65 experiment (bottom right), samples were treated or untreated with RNase A before immunoprecipitation; instead of input, 0.3% of supernatant was western blotted, to control for possible effects of the additional incubation on phosphoepitope availability.

initiation to elongation[45]. Our finding that U2AF65 coimmunoprecipitates with hyperphosphorylated forms of Pol II is consistent with this possibility; however, the sensitivity of this interaction to RNase A treatment suggests that U2AF65 binding to Pol II alone is not stable. Because U2AF65 also coimmunoprecipitates snRNPs, the interaction of U2AF65 with Pol II may be direct or indirect. Thus, the previously observed association of hyperphosphorylated forms of Pol II with snRNPs in splicing reactions *in vitro* may be mediated by U2AF65 (ref. 34,35). U2AF65 also coimmunoprecipitates hypophosphorylated, transcriptionally inactive Pol IIa[32] (data not shown); however, we were unable to detect U2AF65 in promoter regions where Pol IIa is abundant (compare **Figs. 2** and **3**). Therefore, the data lead us to favor the interpretation that U2AF65 binding to nascent RNA is promoted and/or stabilized by cooperative interactions with Pol II. These observations point to major differences in cotranscriptional spliceosome assembly between yeast and mammalian cells.

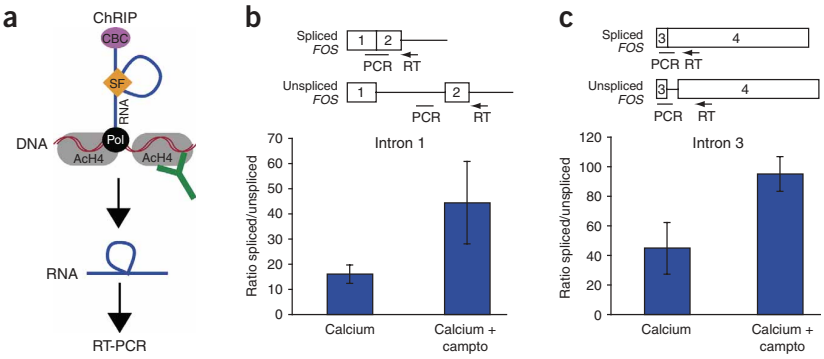

Figure 6 Cotranscriptional *FOS* pre-mRNA splicing detected by ChRIP and enhanced by camptothecin. (**a**) Schematic of experimental approach, illustrating the expectation that antibodies against AcH4 will pull down nascent *FOS* RNA attached to the chromatin through Pol II. (**b**) Ratio of spliced to unspliced *FOS* intron 1 was determined with reverse-transcription (RT) primers in intron 2 and PCR primers amplifying either exon 1–exon 2 or intron 1 (for details, see Methods and **Supplementary Fig. 3**). Note that the placement of the RT primer in intron 2 ensures that potential signals from mRNA do not contaminate the results, though experiments specifically addressing this indicate that nonspecific capture of mRNA is negligible (**Supplementary Fig. 3**). (**c**) Ratio of spliced to unspliced *FOS* intron 3 was determined with RT primers in exon 4 and PCR primers amplifying either exon 3–exon 4 or exon 3–intron 3. Shown are averages and s.e.m. of three independent experiments, in which cells were treated with calcium ionophore for 15 min, followed by an additional 15 min of ionophore with or without camptothecin.

Recent speculation has focused on the possibility that splicing factors, like capping enzymes, may be brought to transcription units by Pol II[1,3,31]. The rationale is that splicing factor binding to Pol II would increase the local concentration of splicing factors at the sites of RNA synthesis and increase splicing efficiency, fidelity or both. However, most splicing factors are expressed in cells at high concentrations, generally in the micromolar range, so this recruitment mechanism may be unnecessary[2]. Indeed, mammalian genes are characterized by poorly conserved and cryptic splice sites, such that locally elevated concentrations might even reduce splicing fidelity. Recent data obtained from *in vitro* systems in which transcription and splicing are coupled show that transcription by Pol II, as opposed to T7 polymerase, leads to higher mRNA abundance and influences alternative splice site selection. Thus, Pol II may mediate the coupling between transcription and splicing[46–48]. Perhaps less abundant splicing factors other than those studied here are directly bound to Pol II, also accounting for the effects of CTD deletion on pre-mRNA splicing[16,49,50]. The present data suggest that intron-containing nascent RNA promotes accumulation of hnRNP A1, U2AF65 and U1 and U5 snRNPs, because only a low abundance of these was detected on the highly transcribed intronless genes, histone *HIST1H2AB* and induced *HSPA1B*, on which elongating Pol II was robustly detectable. If Pol II has a role in recruitment of these factors, relatively low-affinity binding between splicing factors and Pol II must be involved. This is in contrast to the stable interactions observed between the Pol II CTD and capping enzymes and may allow for more flexibility in when and where along a gene splicing factors are stably recruited.

Accumulation of U2AF65, U1 and U5 snRNPs on the induced *FOS* gene was enhanced by treatment with the topoisomerase I inhibitor camptothecin. Notably, the overall distribution of factors along the gene was not altered, and the abundance of Pol II and CBC detected in downstream regions was unchanged by camptothecin. This indicates that the drug stalls the elongating polymerase at various positions along the length of the gene[19,27,28] and allows splicing factors more time to bind the nascent RNA. Camptothecin did not cause splicing factors to accumulate on intronless histone genes or induced heat-shock genes, the latter of which are also camptothecin targets. Therefore, we conclude that the splicing factor signals observed in the presence and absence of camptothecin reflect the potential of splicing factors to bind the nascent RNAs present in the indicated gene regions.

Enhanced splicing factor accumulation on *FOS* in the presence of camptothecin correlated with higher levels of cotranscriptional splicing, providing direct evidence for kinetic competition between transcription and cotranscriptional splicing rates. Alternative pre-mRNA splicing is known to be influenced by promoter identity, transcription rates and the presence of transcriptional pause sites, in a manner consistent with coordination between splicing and transcription[4,51–56]. The fact that constitutive splicing of *FOS* is also influenced by changes in transcription elongation emphasizes that even transcripts with strong splice sites are not fully spliced cotranscriptionally and that further splicing can occur, given additional time before transcription termination. Thus, pre-mRNA splicing occurs in the context of transcription-unit activity and may be regulated within chromatin by diverse cellular mechanisms.

METHODS

Cell culture and treatments. A431 human epidermoid carcinoma cells were grown in DMEM supplemented with 5 mM HEPES (pH 7.2), 100 U ml^{-1} penicillin, 100 μg ml streptomycin and 10% (v/v) FBS. Two hours before FOS induction[19], the medium was replaced with serum-free medium. Cells were either control-treated with 0.2% (v/v) DMSO for 15 min or induced with 5 μM calcium ionophore A23187 (Molecular Probes) for 15 min, and for camptothecin treatment, cells were incubated for an additional 15 min with 10 μM (*S*)-(+)-camptothecin (Sigma). For *HSPA1B* induction, cells were treated with 250 μM sodium (meta)arsenite (Sigma) for 1 h[18].

Antibodies. Monoclonal antibodies specific for RNA polymerase II were purchased from the following companies: 8WG16, Neoclone; H5 and H14, Covance. Monoclonal antibody Pol3/3 against the F domain of Pol II[57] was a gift of D. Eick (GSF Research Centre for Environment and Health). Monoclonal

antibodies CB7 (anti–U1-70K) and (anti–hnRNP A1) were gifts of D.L. Black (University of California at Los Angeles), and MC3 (anti-U2AF65) was from M. Carmo-Fonseca[58] (University of Lisbon) Polyclonal antibodies specific for CBP80 and CBP20 were gifts of E. Izaurralde (European Molecular Biology Laboratory) and U5-116K antiserum was a gift from R. Lührmann (Max Planck Institute of Biophysical Chemistry).

Chromatin immunoprecipitation and real-time PCR. We used a modification of the technique described[59]. Briefly, 10^8 cells were cross-linked with a final concentration of 1% (v/v) formaldehyde added directly to the medium. Cells were washed twice with cold PBS, scraped and collected. Cell pellets were resuspended in 2 ml of SDS lysis buffer (1% (w/v) SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1)) containing complete protease inhibitor cocktail (Roche) and incubated for 10 min on ice. Cell extracts were sonicated with a Branson sonifier W-450 D at 30% amplitude with 15 10-s bursts, resulting in ~500-nt chromatin fragments and then centrifuged for 10 min at 20,817*g*. A 50-µl sample of the supernatant was saved as input DNA and the remainder was diluted 1:10 in ChIP dilution buffer (0.01% (w/v) SDS, 1.1% (v/v) Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HC (pH 8.1), 167 mM NaCl) containing protease inhibitors. The chromatin solution was precleared at 4 °C with sepharose beads for 1 h before overnight incubation (4 °C) with either 10 µg of 8WG16, 20 µg of Pol3/3, 30 µg MC3, 30 µg CB7, 3 µl anti–U5-116K serum, 4 µl anti–hnRNP A1 ascites fluid or 10 µl anti-CBP80 serum. Nonimmune mouse IgG (10–30 µg; Sigma) was used as a control. Complexes were immunoprecipitated with GammaBind G sepharose beads (Pharmacia Biotech) and blocked with 0.2 mg ml^{-1} salmon sperm DNA and 0.5 mg ml^{-1} BSA for 1 h at 4 °C. The beads were washed by rocking for 4 min once in each of the following buffers: low-salt immune complex wash buffer (0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), high-salt immune complex wash buffer (same as previous, but with 500 mM NaCl) and LiCl immune complex wash buffer (0.25 M LiCl, 1% (v/v) NP-40, 1% (w/v) deoxycholic acid, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)); and twice in TE (10 mM Tris-HCl, 1 mM EDTA). The immune complexes were eluted in 1% (w/v) SDS and 50 mM $NaHCO_3$ and cross-links reversed for 6 h at 65 °C. Samples were digested with proteinase K for 1 h at 45 °C and the DNA extracted using the Qiagen PCR purification kit.

DNA templates retrieved by ChIP were analyzed by quantitative real-time PCR on a Stratagene MX3000, using the SYBR Green method (ABsolute QPCR SYBR Green Rox Mix, AB Gene). The reaction volume was 20 µl, with 4 µl DNA template (input 1:10) and 90–900 nM of each primer, according to individual optimization. A cycle of 15 min at 95 °C was followed by 40 cycles of 30 s at 95 °C, 1 min at 60 °C and 30 s at 72 °C, with measuring at the end of the annealing step. Dissociation curves were obtained by heating the samples to 95 °C, cooling them to 55 °C, then heating them to 95 °C with continuous measurement. Primer sets distinguishing between different regions of the genes are available upon request.

The relative proportions of coimmunoprecipitated gene fragments were determined on the basis of the threshold cycle (Ct) for each PCR product. Data sets were normalized to ChIP input values, and then the Ct values obtained from Pol II and splicing factor ChIP were subtracted from the Ct values obtained from templates derived from ChIP with nonspecific antibody, and the fold difference between the two was calculated as $2^{(Ct(nonspec) - Ct(input)) - (Ct(spec) - Ct(input))}$ (ref. 60). The fold difference over background obtained for gene regions was further normalized to the value obtained with a primer pair amplifying an intergenic region on chromosome 10 where no annotated genes could be found. For every gene fragment analyzed, each sample was quantified in duplicate and from at least three independent ChIPs. s.e.m. was determined for each fold difference above the nonimmune control and intergenic control region.

Standard immunoprecipitations and western blot analysis. Whole-cell extracts from semiconfluent A431 cells were prepared in NET-2 buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% (v/v) NP-40) containing protease and phosphatase inhibitors (1 mM NaF, 10 mM β-glycerophosphate). Immunoprecipitation was carried out for 3 h at 4 °C with GammaBind beads coupled to either 10 µl anti-CBC80 serum, 500 µl MC3 or CB7 hybridoma supernatant, or 5 µl anti–U5-116K serum or anti–hnRNP A1 ascites fluid. The beads were washed five times in NET-2 buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% (v/v) NP-40) and proteins were eluted in 50 µl SDS sample buffer. For RNase A digestions, starting extracts were treated with 100 µg ml^{-1} RNase A for 30 min at room temperature. We analyzed 10 µl of immunoprecipitate and 0.3% of starting material by western blot after electrophoresis on a 7.5% SDS-polyacrylamide gel, using H5 or H14. Metabolic labeling was carried out by incubating 15-cm dishes in phosphate-free medium with 1% (v/v) FBS and 100 µCi [^{32}P]orthophosphate overnight, followed by lysis and immunoprecipitation as described above. RNA was extracted from the final pellets with phenol-chloroform, precipitated, resolved on a 10% urea gel and analyzed by phosphorimaging.

Immunoprecipitation of chromatin-RNA complexes. For ChRIP, cells were cross-linked and harvested as in ChIP, but pellets were resuspended in RIPA buffer (50 mM Tris-HCl (pH 7.5), 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.05% (w/v) SDS, 1 mM EDTA, 150 mM NaCl) containing complete protease inhibitors and RNasin (Promega). Extracts were sonicated and insoluble material was pelleted. Supernatant (250 µl) was precleared with sepharose beads for 30 min at 4 °C before addition of 9 µl anti-AcH4 (Upstate) or 10 µg nonimmune IgG for incubation overnight. Immunoprecipitation and washing was done as described for ChIP, and chromatin was eluted for 15 min at 65 °C in 1% (w/v) SDS in TE after proteinase K treatment for 1 h at 45 °C. Cross-links were reversed at 65 °C for 5 h. RNA was extracted with phenol-chloroform and treated extensively with DNase I. complementary DNA was prepared from one-third of the RNA from AcH4 or IgG ChRIPs using Super-Script III (Invitrogen). Primers located in *FOS* intron 2 and exon 4 were used for reverse transcription. The cDNA and no–reverse transcription control were analyzed by quantitative PCR with primers spanning exons 1 and 2 or exons 3 and 4, and intron 1 and exon 3–intron 3, respectively. The relative proportions of coimmunoprecipitated RNA fragments were determined on the basis of the threshold cycle (Ct) for each PCR product. All AcH4 values were at least 3 cycles (that is, eight-fold) more enriched than nonimmune controls. The AcH4 Cts for spliced and unspliced product were subtracted from each other and taken as the exponent of 2 to yield the fold difference for spliced/unspliced: fold difference spliced/unspliced $= 2^{(Ct(unspliced)-Ct(spliced))}$. For every RNA fragment analyzed, each sample was quantified in duplicate and from at least three independent ChRIPs. s.e.m. was determined for each fold difference. Detailed characterization of the method is provided in **Supplementary Figures 2** and **3**.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

K.M.N. and I.L. conceived and designed the experiments. I.L. and A.K.S. performed the experiments. I.L. analyzed the data. K.M.N. wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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