Cleavage and polyadenylation define the 3’ ends of almost all eukaryotic mRNAs and are thought to occur during transcription. We describe a human in vitro system utilizing an immobilized template, in which transcripts in RNA polymerase II elongation complexes are efficiently cleaved and polyadenylated. Because the cleavage rate of free RNA is much slower, we conclude that cleavage is functionally coupled to transcription. Inhibition of positive transcription elongation factor b (P-TEFb) had only a modest negative effect on cleavage, as long as transcripts were long enough to contain the polyadenylation signal. In contrast, removal of the carboxyl-terminal domain of the large subunit of RNA polymerase II had a dramatic negative effect on cleavage. Unexpectedly, the 5’ portion of transcript after cleavage remained associated with the template in a functional, polyadenylation-competent complex. Efficient cleavage required 5’ capping by the human capping enzyme, but the reduction of cleavage seen of transcripts in COOH-terminal domain-less polymerase elongation complexes, was not because of lack of capping.

Processing of eukaryotic mRNA starts during transcription and is influenced by the RNA polymerase II elongation complex (1, 2). Capping, polyadenylation, and splicing have been seen to occur on nascent transcripts in vitro, and a variety of in vivo and in vitro approaches have strongly implicated the carboxyl-terminal domain (CTD) of the large subunit of RNA polymerase II in connecting transcription with these events (3–6). Whereas many of the factors required for mRNA processing have been identified and characterized, much less is known about how the processing and transcription machinery functionally influence each other.

3’ End formation is linked to transcription. Although RNA polymerase II is capable of transcribing hundreds of kilobase pairs in a completely processive manner, after transcribing a functional polyadenylation signal the polymerase usually terminates within 1 kb (3, 7) in a process that requires the CTD (8, 9). Factors required for polyadenylation have been found to associate with isolated transcription complexes (10) and the CTD has been implicated in bringing in the factors (11, 12). There is some controversy about when polyadenylation factors associate with the transcription complex. Polyadenylation factors have been found to associate with promoter binding factors (13), and yeast chromatin immunoprecipitation experiments in one study have localized the factors throughout the gene (11), but in another only at the 3’ end of genes after the passage of a functional polyadenylation signal (14). Recent studies in yeast (15), Drosophila (16), and Xenopus (17) have implicated the CTD kinase positive transcription elongation factor b (P-TEFb) in promoting efficient polyadenylation. Drosophila histone mRNA 3’ end formation, involving a completely different set of factors, was found not to be stimulated by having the RNA in an elongation complex (18). However, a strong transcriptional pause was found at the precise downstream location to allow efficient cleavage suggesting a coordination of transcription and processing that might block read-through transcription into adjacent genes (18).

The discovery of cleavage and polyadenylation factors and the increase in understanding of their mechanisms of action has been driven in large part by advances in the development of increasingly sophisticated in vitro systems. Using isolated nuclei to which a concentrated nuclear extract was added back, it was shown that polyadenylated transcripts could be formed in a cell-free system (19). Four years later, a soluble system using adenovirus transcription complexes isolated from infected cells was demonstrated to carry out accurate 3’ end formation (10). Significantly, soluble systems lacking transcription complexes were developed to study polyadenylation (20) and cleavage (21) and these were instrumental in the identification and purification of the cleavage and polyadenylation factors (22). Whereas it is clear that cleavage and polyadenylation occur co-transcriptionally (17), there are only a few reports of soluble systems that support the two processes. The first soluble transcription system that supported cleavage and polyadenylation utilized HeLa nuclear extract and an adenovirus template (23). Because there was a 1-h lag before 3’ end formation began and because transcription could be inhibited during the next 3 h without affecting processing, the authors suggested that polyadenylation was not coupled to active transcription. Another group demonstrated a coupled system in which cleavage at the bovine growth hormone poly(A) site was stimulated when the transcript was generated by RNA polymerase II compared with T7 polymerase (24). The Proudfoot lab (25) used a similar system to show that transcriptional pausing downstream of a synthetic poly(A) site dramatically stimulated cleavage and polyadenylation of transcripts generated by RNA polymerase II. Although the efficiency of cleavage was low, they were also able to use immobilized templates and found that the 3’ cleavage product remained associated with the template and the 5’ cleavage product was released (26).

To understand mechanistic details of the coupling of RNA processing and transcription, manipulable in vitro systems that demonstrate functional coupling are required. One such human system was used to examine capping during transcription and it was found that the transcription complex had a profound influence on the capping reaction (27). We have adapted that transcription system to the study of polyadenylation and have begun to define parameters of the coupling process.

**EXPERIMENTAL PROCEDURES**

**Construction of the Template**—The DNA construct used for these experiments contained the adenovirus-L3 poly(A) site downstream of a
cytomegalovirus promoter in a pGL2 plasmid (Promega). A 2-kb biotinylated template was generated from this plasmid by PCR using a biotinylated upstream oligo. After removing unincorporated oligos with a Wizard SV Gel and PCR Clean-Up System (Promega), the biotinylated template was bound to streptavidin-conjugated Dynabeads (M-280, Dynal Biotech) as per the manufacturer’s instructions. Transcription from this DNA template yielded a 1.1-kb run-off. When processing was allowed to take place, a cleavage product of 180 nt was generated.

In Vitro Transcription Assays—HeLa nuclear extract (HNE) preparation and immobilized template pulse-chase transcriptions were performed essentially as described previously (28). The usual transcription reaction was 12 μl and contained 20 μM HEPES, 60 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 100 μM S-adenosylmethionine, 1 unit/μl of RNaseOUT (Invitrogen), 1.5 μl of HNE, and 20 ng/μl of template. NTP levels were 500 μM ATP, CTP, and GTP with only labeled UTP (10 μCi/reaction) during the pulse, and all NTPrs were 500 μM during the chase unless indicated differently. P-TEFb was inhibited, where indicated, by the addition of flavopiridol to 1 μM. In some experiments when P-TEFb was inhibited, magnesium was raised to 7 mM during elongation to obtain transcripts of sufficient size for the cleavage reaction. All phases of the transcription reactions were carried out at room temperature (~22 °C). Reactions were done in bulk (multiple reactions in one tube) wherever conditions allowed. In most of the experiments, all lanes came from the same pulse reaction, thus transcription complexes for an entire experiment could be generated in a small volume, and washed in a single tube. Subsequently, they could be split into smaller subsets to allow different conditions (e.g. +/− flavopiridol, +/− chymotrypsin treatment). Importantly, use of an immobilized template allowed us to manipulate the volumes of the various reactions. We found that the best processing occurred by adding 1.5 μl of extract back to elongation complexes isolated from two standard reactions.

Isolation of Complexes, and Further Manipulations—To isolate complexes, short pulses were carried out and transcription was stopped by addition of EDTA to 20 mM. The early elongation complexes (EECs) thus generated were washed 3 times with 200 μl of 20 mM HEPES, 1 mM KCl, and 1% Sarkosyl. These high salt/Sarkosyl wash conditions have been shown to remove all known transcription factors from the polynucleotide template (29). Each wash consisted of a thorough resuspension of the Dynabead-associated complexes in the wash buffer, followed by a 15-s concentration in a Dynal MPC-E concentrator with subsequent removal of supernatant. The high salt/Sarkosyl washes were followed by two additional washes into low salt (60 mM KCl) buffer to yield the starting material for the chases. Although standard chase conditions were 500 μM in each nucleotide, it was found that when P-TEFb was active, optimal processing occurred when ATP was at 500 μM and the other nucleotides were reduced to 50 μM during the chase. This is because the reduced concentrations of nucleotides decreased runoff, and resulted in a higher proportion of transcripts in the size range optimal for processing. Chases were stopped by addition of EDTA to 20 mM. Low salt washes were also performed on the chymotrypsin-treated complexes, to eliminate the trypsin inhibitor. Low salt washes of EECs, even after chymotrypsin treatment, resulted in only minimal losses of transcripts. The polyadenylation assays required further washing of complexes containing longer RNAs to eliminate EDTA and polyvinyl alcohol (PVA) before the poly(A) extension. Washing of these longer complexes resulted in a more substantial, but still small, loss of the transcripts.

Cleavage Reactions—After stopping the chases with EDTA, the cleavage reaction was stimulated about 2–4-fold by adding polyvinyl alcohol (Sigma catalog number 8136). Initially PVA was added to 4%, but later it was found that 2% gave identical results. In the initial search for optimum conditions it was found that addition of 3′-dATP (Cordycepin) or creatine phosphate had no effect on the rate or efficiency of the cleavage reaction. The reactions were incubated in a heat block at 30 °C for the times indicated in the figures. When multiple time points were to be taken out of a bulk cleavage reaction, the tube was removed and quickly agitated to homogeneity by flicking; the aliquot was then taken from the bulk reaction, and the tube immediately returned to the heat block.

Polyadenylation Reactions and Salt Washes—Finished cleavage reactions for which the product was to be polyadenylated or evaluated for salt resistance were washed in 20 mM HEPES, 60–400 mM KCl, 7 mM MgCl₂, and 0.2 mg/ml bovine serum albumin, as follows: double cleavage reactions (30 μl) were pipetted into 175 μl of the indicated salt wash buffer, and gently mixed. The complexes (cleavage products still attached) were then concentrated, the buffer was removed, and an additional 200 μl of buffer was added. The complexes were gently resuspended by pipetting, and concentrated one final time, after which the supernatant was removed, and the complexes were resuspended in a small volume (4–8 μl/reaction) of the 60 mM wash buffer. At this point the RNA was isolated by phenol extraction or subjected to polyadenylation conditions (20 mM HEPES, 60 mM KCl, 7 mM MgCl₂, 500 μM ATP, and 0.2 mg/ml bovine serum albumin). For the experiment shown in Fig. 5, 2.5 μl of HNE was also added in the indicated reactions. All poly(A) extension reactions were stopped with tRNA/Sarkosyl, then phenol extracted and analyzed on a gel.

Chymotrypsin Treatment of Early Elongation Complexes—High salt/Sarkosyl-washed EECs were resuspended in 20 mM HEPES, 60 mM KCl, 7 mM MgCl₂, and 0.2 mg/ml bovine serum albumin. Chymotrypsin was added in the same buffer, to a final concentration of 0.5, or 25 ng/μl, as indicated. Proteolysis was allowed to occur for 10 min at 37 °C, and was terminated by addition of trypsin inhibitor to a final concentration of 50 μg/μl. The treated complexes were then washed 3 times with 200 μl of the buffer.

Antibody Generation and Western Blot—A cDNA-encoding HCE was cloned into pET21a with a COOH-terminal His tag, and recombinant HCE protein was produced in DE3 cells as previously described (27). The full-length His-tagged protein was injected into sheep for the production of antiserum (Elmira Biologicals). Recombinant HCE and titrations of the extract before and after depletion were resolved by SDS-PAGE (8% gel), and transferred to nitrocellulose on a Panther Semidry Electroblotter (Owl Scientific) for 1 h at 500 mA. The membrane was blocked for 10 min in 10% milk, 1× phosphate-buffered saline, 0.1% Tween 20. Incubation with primary antibody was done overnight at 4 °C in 2.5% milk, 1× phosphate-buffered saline, 0.1% Tween 20. Dilution of primary antibody was 1:300. Incubation with secondary antibody (donkey anti-sheep peroxidase conjugate, Sigma) was done at a dilution of 1:20,000 for 2 h at room temperature in 5% milk, 1× phosphate-buffered saline, 0.1% Tween 20. The blot was treated with SuperSignal West Dura Extended Duration Substrate (Pierce) and exposed to film for 4 min. A Biochemi (UVP) imaging system was used to capture an electronic image that was used for quantitation.

Depletion of Nuclear Extract—The sheep antiserum was affinity purified as follows: full-length capping enzyme was immobilized to Actigel-ALD resin (Sterogene) following the manufacturer’s protocol. The antiserum was heated to 56 °C for 30 min, spun in a JS13.1 rotor for 30 min at 13,000 rpm, and additionally passed through a 0.22 luer-lok filter (Millex-GD, polyvinylidene difluoride). The serum was then incubated with the Actigel-immobilized protein for 2.5 h at 4 °C. After washing with phosphate-buffered saline, the specific antibody was eluted with 100 mM glycine (pH 2.5), neutralized with one-fourth volume 500 mM HEPES.
HEPES and 1 mM KCl, and dialyzed against 260 mM HGKE to remove primary amine. The dialyzed antibody was then immobilized to Actigel-ALD resin, again following the manufacturer’s protocol. HeLa nuclear extract was incubated with the immobilized, affinity purified capping enzyme antibody overnight at 4°C, with constant rotation. The resin was then spun out from the extract in a microcentrifuge.

Isolation and Analysis of RNA—Final reactions were ultimately stopped with a large volume (~200 μl) of RNA-Sarkosyl solution. RNA was then phenol-extracted, precipitated with 95% ethanol, 0.5 mM ammonium acetate, and washed once with 70% ethanol. The RNA was resuspended in gel-loading buffer and run on a 6% denaturing gel. After the run, gels were soaked in water for 12–15 min, and dried on a vacuum apparatus before being exposed to film. Dried gels were also analyzed on a Packard InstantImager to obtain quantitative results.

Quantitation and Determination of Cleavage Efficiency—Raw numbers for total counts were obtained on a Packard InstantImager following an overnight acquisition of the image. The InstantImager software (version 2.05) was used to obtain total counts for specific regions of the gels. For each lane, two regions were defined: the “processed” region, which encompassed only the cleaved transcript (or the area in which it would appear), and the “unprocessed” region, which consisted of the area above the processed region, up to and including runoff transcripts. Percent processing was calculated as counts in the processed region divided by total counts in both regions. In each case, the counts processed had a background amount subtracted equal to the counts in the same region of the unprocessed lane. This background subtraction was corrected by a small factor that normalized the totals between each chase/cleavage lane pair, thus accounting for differential loading.

RESULTS

The goal of this study was to develop a human in vitro system to study 3’ end formation through polyadenylation, coupled with transcription. Instead of just requiring the RNA generated by in vitro transcription to be processed in the same reaction, we wanted to demonstrate functional coupling between the transcription apparatus and the processing machinery. This meant we needed to show that the RNA in an elongation complex had an effect on the processing reaction. Because in vitro polyadenylation reactions have been notoriously slow and inefficient compared with the observed rate and efficiency in vivo, one of our goals was to increase the kinetics and the extent of the initial cleavage. To allow manipulation of the transcription and processing conditions, an immobilized template containing the adenovirus L3 poly(A) site inserted downstream of the cytomegalovirus promoter was used (Fig. 1A). The cleavage site was 180 bp downstream of the start site of transcription and was followed by about 900 bp of template that could be used to stall polymerases after they had passed the site of processing. The cytomegalovirus promoter was chosen because it has been extensively used to study the function of the elongation control factors, P-TEFb, NELF, and DSIF (29, 30), a termination factor, TTF2 (31), and the functional coupling of the capping enzyme, HCE, with transcription (27).

Initial Cleavage at a Poly(A) Site Is Functionally Coupled to Transcription—Initial attempts to obtain 3’ end cleavage on T7-generated transcripts containing the adenovirus L3 poly(A) signal in our HNE failed, but we found that accurate cleavage would occur if the transcripts were generated by RNA polymerase II and remained in elongation complexes. Transcription was initiated on the immobilized template during a 45-s pulse following a 10-min preincubation with HNE. The labeled transcripts generated during the pulse were predominately 15 nucleotides in length when analyzed on a denaturing gel (Fig. 1B). These short transcripts are produced because of stalling of the polymerase under the limiting UTP conditions used. During a subsequent 4.5-min chase, the transcripts were elongated with only a small fraction reaching the 1100-nt run-off length. EDTA was added to stop transcription and leave elongation complexes stalled on the template. After addition of PVA to 4%, time points were taken and analyzed. A transcript of the expected size (180 nt) appeared within 5 min and accumulated with time (Fig. 1B, band labeled with *). Quantitation documented that during this time the transcripts below 180 nt in length remained constant and those longer than 180 were reduced concomitant with the increase in the cleaved transcript (data not shown). This demonstrates that our HNE has the factors required for transcription, as well as for cleavage at the poly(A) site.

To investigate the suggested possibility that some polyadenylation factors interact with initiation factors and join the elongation complex during initiation (13), we separated initiation from elongation and cleavage by isolating early EECs. After the pulse, the EECs were repeatedly washed with 1 mM KCl and 1% Sarkosyl to strip all initiation and elongation factors from the elongation complex (28). HNE was added back to the isolated EECs and elongation continued (Fig. 1C). After a 4.5-min chase, EDTA and PVA were added and time points were taken. The 180-nt cleavage product accumulated with similar kinetics to that seen when initiation was not separated from elongation and cleavage (Fig. 1C). We conclude that association of factors during initiation is not required to achieve efficient cleavage.

To prove that cleavage is functionally coupled with transcription we compared the kinetics of cleavage of transcripts in elongation complexes with identical, cleavage-free transcripts. After a 4.5-min chase elongation complexes were split into two equal aliquots. One aliquot was directly subjected to cleavage conditions (as in Fig. 1C) and from the other, the RNA was isolated by phenol extraction, ethanol precipitation, and extensive washing. HNE was added to the isolated RNA, which was then incubated under otherwise identical cleavage conditions. The appearance of the cleaved transcript occurred only with the RNA in elongation complexes, even when the cleavage time for the free RNA was extended to 90 min (Fig. 1D). The kinetics of accumulation of the cleaved transcript indicated that the t1⁄2 for the reaction was less than 10 min when the RNA was in an elongation complex and otherwise did not detectibly occur (Fig. 1E). By this criterion, we conclude that cleavage is functionally coupled to transcription in vitro. Using extracts optimized for cleavage and polyadenylation of exogenously added pre-mRNA substrates, cleavage at the L3 polyadenylation site has been shown to occur with similar kinetics to that seen here in extracts developed for efficient transcription (32). The differences between the ability of the two systems to process exogenous substrates could be because of differences in the length of the unprocessed RNAs (which are significantly longer here) or to the concentration or identity of factors present in the two extracts.

To further characterize the template, we carried out a time course of elongation in the absence of any elongation or processing factors. The EECs generated after a short pulse were washed with high salt and Sarkosyl, and the transcripts were chased in the presence of 500 μM NTPs for up to 32 min. The pattern of transcripts revealed the expected presence of numerous pause sites (Fig. 1F). The strongest pause occurred about 30 nt downstream of the polyadenylation site. Two sites around 150 nt from the start site were found to have a propensity to arrest the polymerase before the polyadenylation site had been reached. It is not clear if the pause and arrest sites found here play any role in polyadenylation, but the strong pause after the polyadenylation signal is reminiscent of the pause sites present downstream of the histone mRNA 3’ processing sites that have been hypothesized to facilitate loading of processing factors (18). Also, in another system arrest sites down-
stream of the cleavage/polyadenylation signal have been shown to play a positive role in coupling transcription with 3' end formation (25, 26).

The Effects of P-TEFb and the CTD on Cleavage—P-TEFb plays an important role in controlling elongation by RNA polymerase II (29) and recent reports point toward it having a positive effect on polyadenylation in *Drosophila* (16) and budding yeast (15), and during transcription and processing of exogenous DNA in *Xenopus* (17). Because of this we developed conditions to try to separate the effect of P-TEFb on elongation from a potential effect on polyadenylation. Elongation and polyadenylation were examined using the assay in which HNE is added back to isolated elongation complexes (as in Fig. 1C). This experimental approach has been shown to successfully reconstitute elongation control by NELF, DSIF, and P-TEFb (30). As expected during a 4-min chase the potent P-TEFb inhibitor, flavopiridol (33), caused a dramatic reduction of run-off transcripts and increased the appearance of short transcripts compared with control reactions (Fig. 2A). The amount of cleaved transcript produced during the processing phase of the reactions was greatly reduced when flavopiridol was included in the reaction, but this could have been because of the reduced level of transcripts long enough to be cleaved. When the chase time in the presence of flavopiridol was doubled, more polymerases made it past the cleavage site and more cleaved transcripts were generated. To examine the situation quantitatively, the percentage cleaved was calculated by dividing the number of counts from cleaved transcripts by the total number of counts from RNA long enough to be processed. These values were plotted versus the time of cleavage (Fig. 2B). Inhibition of P-TEFb by fla-
vopiridol caused about a 3-min lag in the accumulation of cleaved transcripts and a reduction in the extent of cleavage to about 65% of that seen in the control reactions. This small effect of flavopiridol on the processing reaction could be because of the loss of an enhancement of cleavage by P-TEFb or to the lower concentration of elongation complexes containing transcripts long enough to be processed. We have found that cleavage efficiency is dramatically reduced when the concentration of elongation complexes is lowered (data not shown). It is also possible that at the high level of flavopiridol used that other kinases that affect cleavage and polyadenylation could be inhibited. In this regard, inhibition of Cdk7 would have an effect on CTD phosphorylation and on the efficiency of capping (27) and if capping is important for cleavage this could reduce the efficiency of cleavage.

We next examined the role of the CTD in the cleavage reaction. Isolated EECs were treated with 0, 5, or 25 μg/ml chymotrypsin under conditions in which the CTD is preferentially degraded (27, 34). To prove that the elongation complexes were not damaged by the treatment, a 4-min chase without factors was carried out after washing the chymotrypsin away. Little or no effect on the size of the transcripts synthesized was found (Fig. 3A), indicating that the elongation potential of the polymerases was not affected. Because the CTD is required for the effect of P-TEFb on elongation (34), the effect of chymotrypsin treatment of the elongation complexes on P-TEFb-catalyzed phosphorylation was used to demonstrate that the CTD was removed. HNE was added back to the treated complexes followed by a 4-min chase with or without flavopiridol. Inhibition of P-TEFb by flavopiridol had the expected effect on the mock treated complexes. Flavopiridol blocked the synthesis of long transcripts and caused an increase in the short transcripts (Fig. 3A). Treatment of the elongation complexes with either 5 or 25 μg/ml chymotrypsin resulted in a dramatic reduction of the effect of P-TEFb with the average size of transcripts being greatly reduced. As has been seen before (34), removal of the CTD causes a slight reduction in the function of the negative factors resulting in slightly longer transcripts when P-TEFb is inhibited. We conclude that treatment of EECs with 5 μg/ml chymotrypsin is sufficient to remove the CTD, as assayed by loss of P-TEFb function, but does not affect general elongation properties of the polymerase.

We next compared the ability of intact elongation complexes and chymotrypsin-treated complexes to support cleavage. In this experiment, reaction conditions were manipulated to obtain similar amounts of long transcripts after the chase. A 4-min chase of control complexes (CTD intact) with P-TEFb functioning gave the expected pattern of transcripts and efficient cleavage (Fig. 2B). To obtain more long transcripts from control complexes in the presence of flavopiridol, the chase time was increased to 10 min and the MgCl2 was raised to 7 mM, a condition that reduces the effect of negative elongation factors.3 This caused an increase in the amount of cleaved transcript, but quantitation indicated that inhibition of P-TEFb again caused a lag in the cleavage reactions and had a slight effect on the overall extent of cleavage (Fig. 3B). This suggests that the reduction in cleavage seen in the presence of flavopiridol in Fig. 2 was not just because of the reduction in the absolute concentration of elongation complexes, but was rather because of a bona fide effect of P-TEFb (or another flavopiridol-sensitive kinase) on the kinetics of cleavage. When elongation complexes treated with 5 μg/ml chymotrypsin were analyzed under identical conditions, a significant reduction of cleavage was found (Fig. 3, B and C). Cleavage was not eliminated, but the overall extent was reduced to less than 20% that seen with control complexes. We conclude that, unlike inhibition of P-TEFb, removal of the CTD has a dramatic effect on the cleavage reaction.

To gain insight into what transcripts were being used as substrates for the cleavage machinery, a profile analysis of lanes from control reactions before and after 30 min of processing was carried out. At the end of the 4-min chase most of the transcripts were longer than the processed transcript (Fig. 3D). After 30 min the cleaved transcript becomes predominant and the amounts of longer transcripts are reduced. Analysis of the fraction of long transcripts that are cleaved indicated that transcripts that are between 300 and 500 nt are preferentially used as substrates to generate the 180-nt cleaved product. The distribution of cleavage efficiency versus distance transcribed past the cleavage site is a bell-shaped curve with a center around 200 nucleotides downstream.

The Cleaved Transcript and Poly(A) Polymerase Are Associated with the Elongation Complex—To further characterize the coupled transcription and polyadenylation system, the final step of 3′ processing, actual polyadenylation, was examined. Because the cleavage step was carried out in the presence of EDTA it was necessary to either add an excess of MgCl2 or to eliminate the EDTA. Surprisingly, preliminary experiments indicated that the cleaved transcript remained in associa-

3 J. Price and D. Price, unpublished data.
tion with the elongation complex, allowing us to use the latter approach. The standard protocol with add back of HNE to isolated EECs was used to generate the cleaved transcript (Fig. 4A, diagram). The template was concentrated and washed with a buffer containing 60 mM KCl. The bound material is shown as the 0 time point (Fig. 4A). Transcripts of all sizes remained associated with the template, as did the cleaved transcript. As has been found before (35), some short terminated transcripts were found in the supernatant above the concentrated template (data not shown). Because it was not clear if the poly(A) polymerase was still associated with the cleaved transcript, addition of ATP and MgCl$_2$ was complemented with HNE in one set of polyadenylation reactions. Time points up to 2 min were taken and the products were analyzed. Within 10 s, all cleaved transcripts that were going to be polyadenylated had been extended on average about 50 nt (Fig. 4A). These transcripts were synchronously polyadenylated with 200–300 nt added within 2 min. Cleavage products in reactions without readdition of HNE were also polyadenylated with equal efficiency, indicating that poly(A) polymerase was associated with the isolated complexes. There was a slight, but reproducible, difference in the kinetics of polyadenylation with and without HNE readdition. The pattern of transcripts with HNE suggested that the reaction was more synchronous (tighter pattern) at the long time points with the number of As added being more consistent and fewer than without HNE. This could be because of the presence of poly(A)-binding protein, which has been shown to regulate poly(A) tail length (36) and which might not be expected to be found in association with the elongation complex or processing machinery.

Whereas it was not clear how the cleaved transcript remained associated with the template, this association made it possible to determine...
the $K_m$ of poly(A) polymerase for ATP in the functional processing complex. Reactions were carried out except that no labeled nucleotide was used in the generation of the cleaved transcript. After washing the processing complexes associated with the template, labeled ATP (at a constant specific activity) was added at concentrations ranging from 1 to 30 mM, and time points were taken. The labeled transcripts were analyzed by gel, and the quantitative results were plotted (Fig. 4B). A decrease in both counts incorporated and length of the poly(A) tail was found as the concentration of ATP decreased. A double reciprocal plot of $1/v$ versus $1/[ATP]$ for the data from 3 to 30 mM was used to calculate a $K_m$ of 4 mM (Fig. 4C).

To further examine the association of the cleaved transcript and the poly(A) polymerase with the elongation complex, salt sensitivity was determined. The starting material containing the cleaved transcript was generated as before and as diagramed (Fig. 5A). The beads with isolated elongation complexes (and cleaved transcripts) were washed in buffer containing increasing amounts of KCl and then washed back down into 60 mM KCl. There is always some loss of beads during washing and this is indicated by the loss of transcripts below the processed band. The cleaved transcript was efficiently recovered until the salt reached 150 mM and by 200 mM was mostly washed off (Fig. 5A, -ATP lanes). As was found before, after addition of ATP, most cleaved transcripts were polyadenylated after the complexes were washed at 60 mM. However, only half were polyadenylated after the 100 mM KCl wash and none after the 150 mM wash. These findings were quantitated and the percentage of the initial cleaved transcript remaining and the percentage of the initial cleaved transcript that was polyadenylated were plotted versus the salt of the wash (Fig. 5B). Although the amount of cleaved transcript remaining after the wash declined, the fraction of that transcript that was polyadenylated decreased faster. We conclude that the poly(A) polymerase is associated more weakly with the 3' processing machinery than the processing machinery is with the transcription complex.

Requirement of 5' Capping for Coupled Cleavage—Although most 3’ end processing systems utilize capped transcripts to maintain stability of the RNA during long incubations, and the nuclear cap binding complex has been shown to stimulate 3’ end processing in an uncoupled cleavage assay (37), the role of 5’ capping in 3’ end processing has not been studied in detail. To address this issue we generated an antibody to
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25% of that seen when the CTD was intact (Fig. 7). Short transcripts, but this only increased the efficiency of cleavage to allowed capping to occur as evidenced by a decrease in the mobility of caused a reduction of cleavage to 10% (Fig. 7). Addition of more HCE into coupled cleavage reactions. As expected, removal of the CTD effect on capping. To test this, increasing amounts of HCE were added cleavage found when the CTD was removed (Fig. 3) was because of an essential for cleavage, but significantly stimulates the reaction. The recovery of efficient 3' end cleavage was concomitant with the end cleavage was significantly affected by the inhibition of the CTD can influence the kinetics of capping in our system (27). Because flavopiridol may partially inhibit Cdk7, it is possible that the effect of the compound on cleavage is actually because of an indirect effect on capping. This possibility is supported by the finding that inhibition of Cdk7 affects the kinetics of capping (27) in the same way that flavopiridol affects the kinetics of cleavage. Removal of the CTD had a much more dramatic effect by reducing the rate and extent of cleavage 10–20%. The residual cleavage activity could have been because of incomplete removal of the CTD or because of a low level of CTD independent cleavage. The latter possibility was supported by the finding that treatment of the elongation complexes with a wide range of chymotrypsin concentrations all gave the same reduction in efficiency (data not shown). Many studies have pointed toward the CTD playing an important role in polyadenylation (reviewed in Refs. 1 and 3). However, the role of CTD phosphorylation is less clear. In an uncoupled in vitro system RNA polymerase II stimulated cleavage, but it did not matter if the polymerase was phosphorylated or not (40). Polyadenylation was negatively affected in yeast strains lacking the Ser-2 CTD kinase, ctk1 (15), in flies treated with the P-TEFb inhibitor flavopiridol (16), and in injected Xenopus oocytes treated with the P-TEFb inhibitor DRB (17). However, in these in vivo studies it is difficult to quantitatively differen-

FIGURE 5. Salt sensitivity of the association of the cleavage product and poly(A) polymerase. A, transcription, isolation of ECs, add back of HNE, 3' cleavage, washing of products, and polyadenylation, with or without extract, were performed as diagramed on the left and as described under “Experimental Procedures.” Elongation complexes were washed with increasing concentrations of KCl and either analyzed directly (+ ATP), or subjected to 30 s of polyadenylation (+ ATP) before being analyzed. SM, RNA after cleavage used as the starting material. B, quantification of cleavage product remaining and polyadenylation. The percentage of initial cleavage product remaining associated with the washed elongation complexes, as well as the amount that was polyadenylated, were plotted versus the concentration of salt washes.

HCE and used it to deplete the capping enzyme from the HeLa nuclear extract. A comparison of different amounts of HeLa nuclear extract to known amounts of recombinant HCE by Western blot indicated that 1 μl of extract contains about 3 ng of HCE (Fig. 6A). Affinity purified antibodies to HCE were covalently coupled to beads and used to remove HCE from an aliquot of the extract. After the depletion procedure HCE was reduced to less than 15% as calculated by quantitative Western blotting using actin as a loading control (Fig. 6A). A comparison of the coupled cleavage reactions carried out in the presence of intact or HCE-depleted extract demonstrated a reduction in cleavage efficiency to 35% seen after 30 min (Fig. 6B). Supplementation of the depleted extract with recombinant HCE allowed recovery of cleavage to almost normal levels (80–90%) (Fig. 6, B and C). We used the fact that capping causes a decrease in mobility of transcripts during gel electrophoresis to monitor capping and found that addition of capping enzyme lead to capping when the level of HCE reached that normally found in the extract (2–6 ng). The recovery of efficient 3' end cleavage was concomitant with the recovery of capping (Fig. 6, B and C). We conclude that capping is not essential for cleavage, but significantly stimulates the reaction.

Because the removal of the CTD slows the kinetics of capping enzyme function in our system (27), we wondered if the decrease in 3' end cleavage found when the CTD was removed (Fig. 3) was because of an effect on capping. To test this, increasing amounts of HCE were added into coupled cleavage reactions. As expected, removal of the CTD caused a reduction of cleavage to 10% (Fig. 7). Addition of more HCE allowed capping to occur as evidenced by a decrease in the mobility of short transcripts, but this only increased the efficiency of cleavage to 25% of that seen when the CTD was intact (Fig. 7B). We conclude that the CTD plays a role in 3' end cleavage above and beyond the stimulation of capping.

DISCUSSION

We have developed and characterized an in vitro system in which cleavage and polyadenylation are functionally coupled to transcription in several ways. Importantly, the rate of cleavage at the poly(A) addition site was dramatically increased by the presence of the RNA in an elongation complex. RNA in an elongation complex was efficiently cleaved with a t1/2 of less than 10 min, whereas under the same extract conditions, identical free RNA was not processed to any appreciable extent, even after 90 min. This functional coupling of transcription to cleavage depended on the CTD, but was only modestly affected by the inhibition of the kinase activity of P-TEFb with flavopiridol. Analysis of the RNA before and after cleavage indicated that RNA with 3' ends between 100 and 300 nucleotides downstream of the cleavage site were preferentially cleaved. Surprisingly, the 5' cleavage product remained in association with the elongation complex and was rapidly and efficiently polyadenylated by poly(A) polymerase that is also associated with the elongation complex. We also found that 5' end capping had a positive effect on 3' end cleavage and that the CTD has a positive effect on 3' end cleavage in addition to its effect on capping.

The CTD and P-TEFb were found to influence both the rate and efficiency of cleavage in our coupled system. When P-TEFb was inhibited the major effect was to reduce the number of transcripts long enough to be processed. This is consistent with the well documented effect of P-TEFb on transcription elongation (29, 34, 38). Inhibition of P-TEFb leaves many RNA polymerase II elongation complexes under the influence of negative transcription elongation factors and traps them in promoter proximal positions on the template in a process termed abortive elongation (39). When chase times were greatly extended and MgCl2 levels raised from 5 to 7 mM to allow elongation in the absence of P-TEFb function, transcript length increased and cleavage was again fairly robust. Under these conditions kinetic analysis indicated that the effect of P-TEFb inhibition on cleavage was to reduce the overall extent to about 65% of that seen when P-TEFb was functional. There was also a noticeable lag in cleavage seen during the first 3 min of the reaction. This could be because of a reduction in the rate of association of a functional cleavage complex or because of the lack of an activation function for the cleavage factors. Cdk7 phosphorylation of the CTD can influence the kinetics of capping in our in vitro system (27). Because flavopiridol may partially inhibit Cdk7, it is possible that the effect of the compound on cleavage is actually because of an indirect effect on capping. This possibility is supported by the finding that inhibition of Cdk7 affects the kinetics of capping (27) in the same way that flavopiridol affects the kinetics of cleavage. Removal of the CTD had a much more dramatic effect by reducing the rate and extent of cleavage to 10–20%. The residual cleavage activity could have been because of incomplete removal of the CTD or because of a low level of CTD independent cleavage. The latter possibility was supported by the finding that treatment of the elongation complexes with a wide range of chymotrypsin concentrations all gave the same reduction in efficiency (data not shown). Many studies have pointed toward the CTD playing an important role in polyadenylation (reviewed in Refs. 1 and 3). However, the role of CTD phosphorylation is less clear. In an uncoupled in vitro system RNA polymerase II stimulated cleavage, but it did not matter if the polymerase was phosphorylated or not (40). Polyadenylation was negatively affected in yeast strains lacking the Ser-2 CTD kinase, ctk1 (15), in flies treated with the P-TEFb inhibitor flavopiridol (16), and in injected Xenopus oocytes treated with the P-TEFb inhibitor DRB (17). However, in these in vivo studies it is difficult to quantitatively differen-
associated with the elongation complex. How these associations were because both the poly(A) polymerase and the cleaved transcript were observed may be accomplished through association of CPSF with the elongation complex, the RNA with CPSF160 and Fip1, and the poly(A) script and poly(A) polymerase with the elongation complex that we observed to stimulate polyadenylation (42). The association of the cleaved transcript, suggesting that poly(A) polymerase does not strongly associate with RNA, but cleavage and polyadenylation factor CPSF does. CPSF160 binds to the AAUAAA signal, but does not stimulate poly(A) polymerase (41). Another CPSF subunit, Fip1, binds to RNA and poly(A) polymerase, and enables CPSF to stimulate polyadenylation (42). The association of the cleaved transcript and poly(A) polymerase with the elongation complex that we observed was accomplished through association of CPSF with the elongation complex, the RNA with CPSF160 and Fip1, and the poly(A) polymerase through Fip1. Our findings suggest that the interaction of poly(A) polymerase with the elongation complex is weaker than the association of the cleaved transcript, suggesting that poly(A) polymerase does not contribute significantly to the association of the cleaved transcript. The $K_m$ of 4 $\mu M$ we determined for poly(A) polymerase toward ATP was significantly lower than the 228 $\mu M$ found with purified bovine enzyme in a distributive assay uncoupled from a transcription (43). This decrease in $K_m$ could be because of an allosteric effect of the cleavage complex on the function of poly(A) polymerase or because of an increase in the local concentration of the RNA substrate through the interactions discussed above.

We examined the effect of capping on 3' end formation and found that there was a significant stimulation of cleavage by the human capping enzyme. Because under the conditions used there is little or no turnover of RNA, the effect was not because of stabilization of the transcript. The positive effect of HCE on cleavage was most likely because of capping, rather than to a non-catalytic effect of the HCE protein, because the capping enzyme does not travel with the polymerase into the 3' end of genes (44). The Gilmartin laboratory (37) has shown that the nuclear cap binding complex has a positive effect on cleavage using an uncoupled in vitro system, but it is not clear from their study if the 5' cap is associated with the cap binding complex. If it is, it is possible that the mRNA forms a loop in which both the 5' to 3' ends of the mRNA are associated with the elongation complex. Consistent with this we have found that the 5' cleaved transcript is associated with the elongation complex after cleavage. This is likely because of association of the 3' end of the 5' cleaved transcript with the polyadenylation machinery, but the
interaction could also be stabilized by interaction of the 5’ cap with the cap binding complex, which in turn interacts with the processing machinery associated with the elongation complex.

Termination and 3’ end formation are linked in vivo, but in our in vitro system we were not set up to detect termination associated with cleavage. We assume that termination did not occur because the cleaved transcript remained associated with the immobilized template. It is possible that after polyadenylation the polymerase was released from the template or it is possible that termination does not occur under the conditions used here, which stall the polymerase by removal of NTPs. The exact timing of cleavage, polyadenylation, and termination in vitro is still unclear (45). Recent experiments indicate that extended communication with the AAUAAA signal is needed (9). We favor a model in which the AAUAAA signal triggers a transition of the elongation complex to re-enter abortive elongation. This could be through Ser-2 dephosphorylation of the CTD. The cleavage factors could then be associated in a phosphorylation independent way and lead to cleavage and polyadenylation. Ser-2 phosphorylation by P-TEFb was shown here to stimulate an uncoupled cleavage reaction (40). Termination could then occur through the function of the 5’ to 3’ exonuclease (46, 47) or possibly through the ATP-dependent function of TTF2 (48).

The coupled system described here will be useful in elucidating more mechanistic details of cleavage and polyadenylation as they occur in the normal context of an elongation complex. The sequence requirements of the cleavage signals and the position effects of those sequences relative to the start site of transcription could be examined. Because the template is immobilized it should be possible to examine the timing of the association of the cleavage and polyadenylation factors and determine how the cleaved transcript is held on the elongation complex. It also may be possible to determine the factor requirement for termination in conjunction with cleavage and polyadenylation, if conditions can be found that allow termination.

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