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Solid-Phase Processing of U2 snRNA Precursors†

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ABSTRACT: HeLa cell cytoplasmic extracts contain both precursors to small nuclear RNA (snRNA) U2 and an activity that is capable of trimming these snRNA precursors to the size of mature U2. The substrate for this RNA processing reaction is the ribonucleoprotein complex containing pre-U2 RNA. To circumvent the difficulty of biochemically isolating pre-U2 ribonucleoprotein (pre-U2 RNP) complexes for use as substrate for the analysis of the processing activity, we have developed a procedure for the processing of pre-U2 RNP complexes that have been immobilized on anti-Sm antibody/protein A–Sepharose columns. When the immobilized [^{3}H]uridine-labeled substrate RNP complexes are incubated at 37 °C with unlabeled cytoplasmic extracts from HeLa cells, labeled molecules the size of mature U2 are produced in a linear fashion for up to 3 h. Similar results are obtained when substrate pre-U2 RNPs are immobilized with an anti-2,2,7-trimethylguanosine antibody. Thus, accurate processing of the 3′ termini of U2 precursors occurs on the antibody columns. Incubation with buffer alone does not result in the production of mature-sized U2, indicating that the processing activity is not intrinsic to the pre-U2 RNP. Using this assay procedure, we have demonstrated that the processing activity is destroyed by trypsin or by preincubation at 65 °C but is resistant to treatment with micrococcal nuclease. These results are compatible with the conclusion that the processing activity is a classical enzyme that does not contain a nuclease-sensitive essential RNA component.

U2 RNA is one of a family of small nuclear RNAs found in eukaryotic cells. Recent evidence supports the hypothesis that U2 plays an important role in RNA splicing (Black et al., 1985; Krainer & Maniatis, 1985; Calvet et al., 1982). In HeLa cells, U2 is synthesized as a precursor that is approximately 10 nucleotides longer than the mature species (Elicieri & Sayavedra, 1976; Wieben et al., 1985; Yuo et al., 1985). Since both U2 precursors and mature U2 snRNAs[1] are complexed with the proteins which confer antigenicity with respect to autoimmune sera, it seems likely that the substrate for the processing reactions is the ribonucleoprotein complex rather than naked snRNA. Our recent experiments investigating the processing of pre-U2 RNAs in vitro support this idea.

Precursors to small nuclear RNA U2 that are present in HeLa cell cytoplasmic extracts are efficiently processed to capped RNAs the size of mature U2 upon incubation of the extract at 37 °C. Under these conditions, exogenously supplied naked U2 precursors are rapidly degraded, suggesting that the ribonucleoprotein structure of the endogenous pre-U2 RNA plays at least a passive role in the correct processing of U2 (Wieben et al., 1985). While raising interesting questions concerning the role of the snRNP proteins in the processing of snRNA precursors, this finding poses a significant technical problem for the study of this activity: that of purifying unprocessed pre-U2 RNA which retains sufficient RNP structure to be accurately processed in vitro. We have circumvented this problem and developed a simple and effective assay for U2 processing activity. We have used this assay to further characterize the U2 processing activity.

Materials and Methods

Preparation of Cell Extracts and Endogenous Processing Reactions. HeLa cells were grown in suspension culture and pulse-labeled for 45 min with [^{3}H]uridine as described. Cells were fractionated in 0.01 M NaCl, 1.5 mM $MgCl_2$, and 0.01 M Tris-HCl, pH 7.5 (RSB). The cytoplasmic fraction was removed and adjusted to either 0.1 M NaCl, 2.5 mM $MgCl_2$, and 0.01 M Tris-HCl, pH 8.5 (pH 8.5 buffer), for direct antibody selection or 0.11 M NaCl, 6.5 mM $MgCl_2$, and 0.02 M Tris-HCl, pH 8.0 (adjusted RSB), for endogenous processing reactions, which were performed as described previously (Wieben et al., 1985).

Antibody Selections. Antibody selections were performed as described (Wieben et al., 1985). Anti-2,2,7-trimethylguanosine or patient anti-Sm antisera was incubated with cytoplasmic samples for 60 min on ice, followed by the addition of 50 μL of protein A–Sepharose. After an additional 60-min incubation, 0.4 mL of carrier Sepharose CL-4B was added,

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[1] Abbreviations: snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein.

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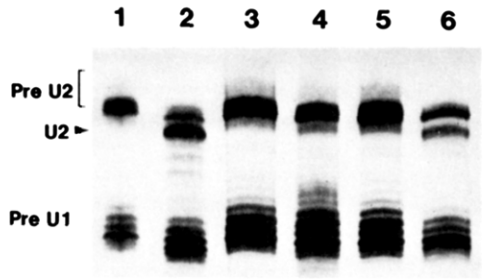

FIGURE 1: Solid-phase processing of pre-U2 snRNP. A sample of 250 mL of HeLa cells was concentrated 10-fold and incubated at 37 °C for 45 min with 5 mCi of [^{3}H]uridine. A cytoplasmic extract was prepared and either used directly for analysis of U2 processing activity of endogenous snRNPs in solution (lanes 1 and 2) or reacted with an Sm autoantibody in preparation for solid-phase processing (lanes 4–6). Lanes 1 and 2: processing of endogenous U2 snRNP in cytoplasmic extracts. Incubation was for 90 min at 4 °C (lane 1) and at 37 °C (lane 2). Lanes 3–6: solid-phase processing of U2 snRNA. Lane 3: buffer only, incubated at 4 °C. Lane 4: buffer only, incubated at 37 °C. Lane 5: cytoplasmic extract, incubated at 4 °C. Lane 6: cytoplasmic extract, incubated at 37 °C.

and the entire reaction was transferred to a 0.5 × 4 cm column for washing. Columns were washed with 0.15 M NaCl, 5 mM EDTA, 0.05 M Tris-HCl, pH 7.5, and 0.5% Nonidet P-40 (Net 2 buffer), followed by additional washing with adjusted RSB.

Solid-Phase Processing Reactions. The solid-phase processing reactions were initiated by the addition of unlabeled cytoplasmic extract from HeLa cells (in adjusted RSB) to washed columns containing pulse-labeled snRNPs. After incubation, the columns were washed with Net 2 a final time and eluted with 0.1 M glycine, pH 3.0, and 1% SDS. RNA was prepared from the eluted samples by phenol extraction and electrophoresed on 7 M urea and 10% polyacrylamide gels as described previously (Wieben et al., 1985). Specific variations to this protocol are detailed in the figure legends.

RESULTS

An example of the activity of the HeLa extract in processing endogenous U2 precursors is shown in lanes 1 and 2 of Figure 1. As reported previously, pre-U2 RNAs are efficiently converted to mature U2 by this procedure. To develop an assay system for the U2 processing activity which would be active on exogenously supplied U2 precursors, we took advantage of our serendipitous observation that the processing of U2 precursors is not inhibited by the prior addition of anti-Sm or anti-trimethylguanosine cap antisera. Substrate pulse-labeled pre-U2 snRNPs (along with those for U1 and U4) are isolated on immunoaffinity columns, and the matrix-bound snRNPs were incubated with unlabeled cytoplasmic extract adjusted to 0.11 M NaCl, 6.5 mM $MgCl_2$, and 0.02 M Tris-HCl, pH 8.0. When the incubation is performed at 37 °C, significant amounts of mature-sized U2 RNAs accumulate within 45 min (compare lanes 4 and 6 of Figure 1). Lanes 3 and 5 of Figure 1 demonstrate that no products are detected when the incubation is performed at 4 °C. Concomitant with the production of processed U2, there is some shortening of the ladder of pre-U1 species during incubation with unlabeled extract at 37 °C. This is a highly reproducible observation and may be indicative of some action of processing enzymes on a subset of U1 precursors. Significantly, incubation of the matrix-bound pre-U2 snRNPs with buffer alone at 37 °C (lane 4) does not result in appreciable conversion to molecules the size of mature U2. This result indicates that the U2 processing reaction is not autocatalytic. Additionally, it suggests that the snRNP proteins associated with the pre-U2

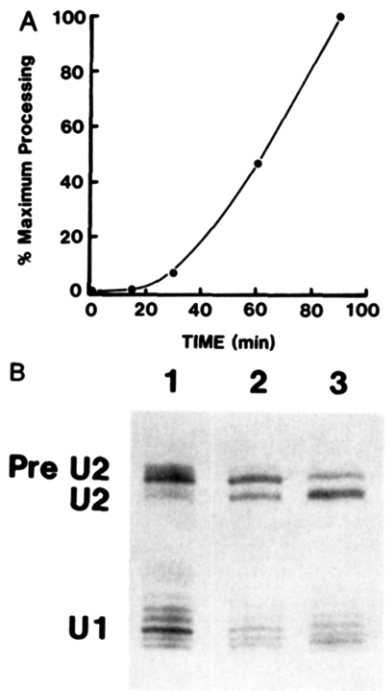

FIGURE 2: (A) Time course of solid-phase processing reaction. HeLa cells were pulse-labeled for 45 min with [^{3}H]uridine. Equal aliquots of cytoplasmic extract were incubated with anti-Sm antibody and protein A–Sepharose as described in Materials and Methods. Solid-phase processing of isolated snRNPs was initiated by the addition of prewarmed unlabeled cytoplasmic extract to the matrix-bound snRNPs. After the indicated times, the columns containing the substrate snRNPs were then washed and eluted, and the resultant mixture was prepared for electrophoresis as described previously. The dried gel was exposed to preflashed X-ray film for 3 days, and the processing reaction was quantitated by scanning densitometry. The percent processing is defined as the percentage of the total population of U2 RNAs that are converted to molecules the size of mature U2. The maximum amount of processing obtained in this experiment was 44%, shown here as 100% at 90-min maximal activity. A background of 11% processing (obtained by incubation of matrix-bound snRNPs at 37 °C for 90 min in buffer alone) was subtracted from each of the data points shown. (B) Solid-phase processing continuing for up to 3 h. Conditions were the same as in (A) except a different cytoplasmic extract was used in the experiments shown in (B). All samples were incubated at 37 °C. Lane 1: buffer only, incubated for 3 h. Lane 2: cytoplasmic extract, incubated for 90 min. Lane 3: cytoplasmic extract, incubated for 3 h.

RNA (Wieben et al., 1985) are not themselves responsible for the trimming of the U2 precursors.

It is noteworthy that the solid-phase processing of U2 precursors is not as efficient as the processing of endogenous U2 substrates (compare lanes 2 and 6 of Figure 1). This difference is apparently due to the reduced rate of processing of the matrix-bound substrate. While the processing of the pre-U2 species in the HeLa extract is essentially complete after 25 min at 37 °C, only a few percent of the matrix-bound precursors are processed in the same period (Figure 2). However, after the initial lag period, the production of mature U2 molecules is linear for at least 1 additional h. Further experiments (Figure 2B) have established that under conditions of enzyme excess, processed molecules continue to be produced for up to 3 h of incubation at 37 °C.

Figure 3 demonstrates that the success of this procedure is not dependent upon the usage of anti-Sm antibody. Pre-U2 snRNPs immobilized on protein A–Sepharose via anti-2,2,7-trimethylguanosine cap antibody (Luhrmann et al., 1982) (lanes 4 and 5) can also be converted to mature U2-sized molecules during incubation at 37 °C with HeLa cytoplasmic extract. Since the retention of the U2 molecules on the matrix

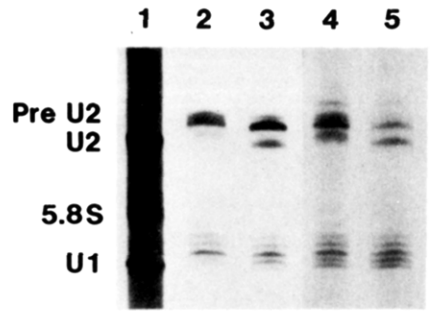

FIGURE 3: Production of labeled RNA the size of mature U2 is not inhibited when anti-2,2,7-trimethylguanosine cap antibody is used for the immobilization of pre-U2 snRNPs. snRNP samples were prepared as in Figure 1 except in lanes 4 and 5 where a cap antibody was used to immobilize the snRNPs instead of an Sm antibody. Samples were incubated for 90 min at 37 °C. Lane 1: [^{3}H]-uridine-labeled nuclear snRNPs (markers). Lane 2: anti-Sm, incubated with buffer only. Lane 3: anti-Sm, incubated with cytoplasmic extract. Lane 4: anti-trimethylguanosine cap antibody incubated with buffer only. Lane 5: anti-trimethylguanosine cap antibody, incubated with cytoplasmic extract.

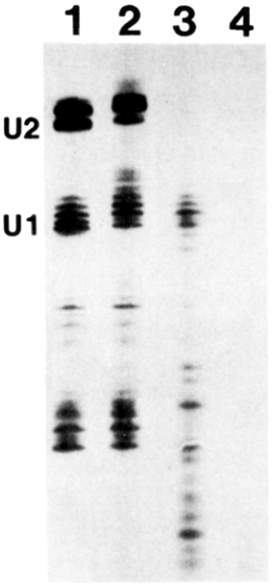

FIGURE 4: Processing reaction cannot be mimicked by incubation with nonspecific endonucleases. Labeled snRNP samples were prepared and immobilized on protein A–Sepharose columns as described in Figure 1. All columns were incubated at 37 °C for 90 min. Incubation was with either unlabeled cytoplasmic extract (lane 1), buffer only (lane 2), buffer plus 0.004 μg of RNase A and 0.0002 μg of RNase T1 (lane 3), or buffer plus 0.04 μg of RNase A and 0.002 μg of RNase T1 (lane 4).

is dependent upon the continued integrity of the 5′ end of U2 during processing, we infer that processing is occurring at the 3′ terminus. The production of capped molecules the size of mature U2 strongly suggests that the processing event being monitored is faithful and accurate.

One possible role for the protein components of the substrate RNP is to protect the mature U2 sequences from degradation during the processing reaction. In fact, it is possible that the entire processing reaction could be accomplished by nonspecific nucleases acting on a pre-U2 molecule which is covered by protein except at the 3′ tail. To evaluate this possibility, immobilized pre-snRNPs were incubated with increasing concentrations of RNases A and T1. After 90 min of incubation at 37 °C, the RNases were removed by washing the column with buffer, and the RNA products were recovered by extraction with phenol. Although the higher concentrations of nuclease resulted in the complete degradation of all RNAs

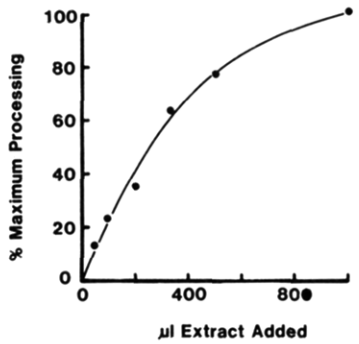

FIGURE 5: Processing of matrix-bound pre-U2 snRNPs by HeLa cytoplasmic extract. The matrix-bound [^{3}H]uridine-labeled snRNPs were incubated for 45 min at 37 °C with increasing amounts of unlabeled HeLa cell cytoplastic extract. The total volume of cytoplasmic extract plus buffer in each case was 1 mL. After the incubation period, the columns were washed again, and the products were eluted with 0.1 M glycine, pH 3.0. RNAs were isolated by phenol extraction and ethanol precipitation. Products were analyzed by electrophoresis and fluorography. The dried gel was exposed to preflashed X-ray film for 3 days, and the processing reaction was quantitated by scanning of the film with a scanning densitometer. The percent processing is defined as the percentage of the total population of U2 RNAs that are converted to molecules the size of mature U2. The maximum amount of processing obtained in the experiment was 24%. A background of 4% processing (obtained by incubation of matrix-bound snRNPs at 37 °C in processing buffer alone) was subtracted from each of the data points shown.

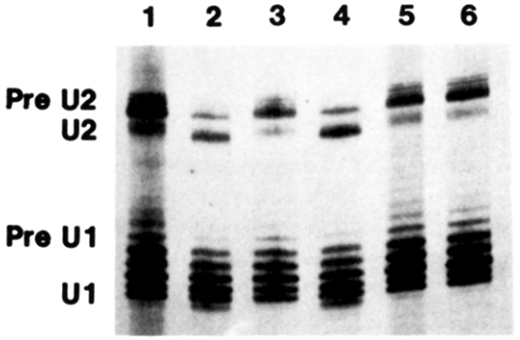

FIGURE 6: Inactivation studies. Labeled pre-U2 snRNPs were prepared as in Figure 1. Incubations were for 90 min at 37 °C in all cases. Where indicated, the unlabeled cytoplasmic extract was treated as described before application to the columns containing labeled substrate snRNPs. Lane 1: buffer only control. Lane 2: HeLa cytoplasmic extract. Lane 3: HeLa cytoplasmic extract adjusted to 0.1 M $MgCl_2$. Lane 4: HeLa cytoplasmic extract (1 mL) supplemented with 1 mM $CaCl_2$ treated with 150 units of micrococcal nuclease for 15 min at 20 °C followed by inactivation of the nuclease with 2 mM EGTA. Lane 5: HeLa cytoplasmic extract treated with 10 μg of trypsin followed by inactivation of the trypsin with 10 μg of soybean trypsin inhibitor. Lane 6: HeLa cytoplasmic extract heated to 65 °C for 30 min and cleared by centrifugation prior to application to the column containing snRNPs.

(lane 4, Figure 4), some resistant RNAs were evident at lower nuclease concentrations. Significantly, at nuclease concentrations that only marginally affect pre-U1 species, no molecules the size of pre-U2 or mature U2 RNA are recovered (lane 3, Figure 4). This suggests that the pre-U2 molecule is accessible to nucleases at sites other than the 3′ tail and argues against a purely passive role for the RNP proteins in the processing reaction.

The effectiveness of this procedure for assaying U2 processing activity is demonstrated by Figure 5. The incubation of a fixed amount of matrix-immobilized pre-U2 with increasing amounts of HeLa cytoplasmic extract results in the production of increasing amounts of processed U2. Thus, this procedure can be used to assay the relative amounts of U2 processing activity present in different fractions.

Using this assay protocol, we have begun to characterize the processing activity. Figure 6 shows that the processing activity is inhibited by high magnesium concentrations (0.1 M, lane 3) and is destroyed by pretreatment of the HeLa cytoplasmic extract with heat (65 °C for 30 min, lane 6) or trypsin (100 μg/mL, lane 5). Interestingly, the processing activity described here is completely resistant to pretreatment with micrococcal nuclease at concentrations of up to 1500 units/mL. Lane 4 of Figure 6 depicts the results of pretreating the cytoplasmic extractant with 150 units/mL of micrococcal nuclease for 15 min at 20 °C. Identical results are obtained when nuclease concentrations up to 10-fold higher (1500 units/mL) are used (data not shown). These results are compatible with the hypothesis that the processing activity resides in a classical enzyme lacking a nuclease-sensitive nucleic acid component.

Discussion

Like most other cellular RNAs, small nuclear RNAs are synthesized as precursors. Following the assembly of pre-snRNAs with specific snRNP proteins, the RNAs are processed to produce the stable nuclear species that are involved in RNA splicing. This order of events dictates that the natural substrate for the pre-snRNA processing reaction is a ribonucleoprotein complex. Our previous study established that the RNA component of pre-U2 RNP complexes is converted to mature U2 when cytoplasmic extracts of HeLa cells are incubated at 37 °C. No further characterization of the processing reaction was possible using that procedure, since both the substrate RNP and the processing machinery were present in the HeLa extract. A further limitation of the previous processing procedure was imposed by the finding that exogenous, naked U2 precursors were rapidly degraded by the cytoplasmic extracts. Thus, at least for the assay of crude extracts, a procedure that permits the determination of the processing activity of exogenously supplied pre-U2 RNPs was required. We have now developed an assay for U2 processing activity which permits further analysis and fractionation of the active components. The present work establishes that pulse-labeled pre-U2 RNPs immobilized on protein A–Sepharose columns are adequate substrates for processing by crude extracts. This procedure is rapid and exhibits good sensitivity and linearity of response to increasing amounts of enzyme activity. The major source of variability in the assay procedure is in the preparation of the cytoplasmic extract. Different extracts vary by as much as two-fold with respect to their titer of U2 processing activity. [This parallels the extent of variation observed in the activity of HeLa nuclear extracts in RNA splicing (Krainer et al., 1984).] However, the quantitative data presented in Figures 2 and 5 demonstrate that only minimal variability is experienced when this assay is used to evaluate the U2-processing activity of a given extract. Therefore, this procedure can be used as a semiquantitative assay to characterize and isolate the active component(s) of the processing activity.

The use of this improved assay system has allowed us to characterize the U2 processing activity in greater detail. The experiments reported here are most consistent with the hypothesis that U2 is processed by a classical protein enzyme, which is sensitive to both trypsin treatment and heat inactivation. If the processing activity does have an essential RNA component, then it is relatively insensitive to digestion by micrococcal nuclease. The nuclease concentrations used in our experiments are in excess of those required to completely abolish the splicing activity of nuclear extracts (Krainer & Maniatis, 1985). Comparable amounts of nuclease are also sufficient to eliminate the activity of RNase P (Stark et al., 1978) and to eliminate endogenous mRNA from reticulocyte lysates (Pelham & Jackson, 1976). However, recent experiments have demonstrated that the 5′ end of U5 RNA is resistant to nuclease treatment even at significantly higher enzyme concentrations (10000 units/mL; Chabot et al., 1985). Hence, we cannot rule out the involvement of a small RNA component in the processing of U2 precursors.

On the basis of in vitro mutagenesis experiments, Yuo et al. (1985) suggested that a U2 specific protein might be involved in the final processing of U2 precursors. Previous experiments using an anti-(U1)(U2) antiserum suggest that this protein is included in the proteins which are bound to pre-U2 under the conditions used (Mattaj & DeRobertis, 1985; Madore et al., 1984). However, since isolated pre-U2 snRNPs do not produce detectable amounts of mature U2 when incubated under processing conditions, it seems unlikely that any of the proteins which are associated with pre-U2 are themselves responsible for the processing reaction. Furthermore, our results suggest that the protein components of the pre-U2 snRNP do not function merely to protect the mature U2 sequence from digestion by nonspecific endonucleases. Pre-U2 RNP was significantly more sensitive to digestion by RNases A and T1 than pre-U1. The use of endonucleases for this experiment was dictated by the pattern of products seen after shorter intervals of processing. Despite the fact that the processing of the immobilized substrate is quite slow, there is no evidence for discreet processing intermediates such as might occur during the processive digestion of the 3′ end of pre-U2 by an exonuclease (as there is for pre-U1). In this processing system and in *Xenopus* oocytes (Yuo et al., 1985), no major bands are detected between the +10 pre-U2 and the band of mature U2. Nor is there any evidence for nibbling of an exonuclease at the 3′ end of mature U2 as might be expected if the extent of processing were limited strictly by protein protection. It is worth noting in this regard that M5, the enzyme responsible for the terminal processing of 5S RNA in *Escherichia coli*, is an endonuclease (Stahl et al., 1984; Pace et al., 1984). Of course, despite these considerations, it remains possible that nonspecific exonucleases are responsible for U2 processing and that the snRNP proteins are required to limit the extent of this reaction.

A more complete understanding of why the U2 processing reaction requires snRNP proteins will emerge from continued study of more purified preparations of the processing activity. By comparing the activity of purified preparations toward both snRNP and snRNA substrates, it should be possible to determine which, if any of the protein components of the pre-snRNP have an obligatory role in the processing of U2.

Acknowledgments

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The σ Subunit of RNA Polymerase Contacts the Leading Ends of Transcripts 9–13 Bases Long on the λ P_R Promoter but Not on T7 A1[†]

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ABSTRACT: The σ subunit of RNA polymerase is responsible for specific initiation of RNA synthesis at promoter sites on DNA. σ dissociates shortly after initiation. Photoaffinity-labeling experiments performed on transcription complexes with two different DNA promoters, which have highly homologous control sequences upstream from the transcribed regions, have revealed that the σ subunit of RNA polymerase is contacted by the 5′ ends of quite different lengths of nascent RNA in each transcription complex. On the other hand, the labeling of subunits $\beta\beta'$ is quite similar for both promoters, and the α subunit is not labeled in either case. The results of transcription experiments on the phage λ P_R promoter show that σ can be photoaffinity labeled by RNA chains that are 9–13 nucleotides long and thus remains associated with the core enzyme at least to that point. But on the A1 promoter of phage T7 DNA, photoaffinity labeling of σ ceases with the trinucleotide. Thus release of σ from the vicinity of nascent RNA depends not merely on the length but on the sequence of the transcript. For the T7 A1 promoter, σ labeling ceases while the leading end of the RNA is still base paired to the DNA template; thus, it appears that there is at least one site on the enzyme that interacts with the growing transcript/template hybrid, in a sequence-dependent way, to effect σ release. Similarities with the results of Hansen and McClure [Hansen, U. M., & McClure, W. R. (1980) *J. Biol. Chem. 255*, 9564–9570] for σ dissociation from transcription complexes on poly[d(A-T)] are discussed, with the observation that the RNA/DNA hybrids formed on T7 A1 show an alternating pattern of bridging hydrogen bond donors and acceptors in the major groove that exactly matches the one formed on poly[d(A-T)]. This property is not shared by λ P_R.

DNA-dependent RNA polymerases catalyze the synthesis of ribonucleic acid, with deoxyribonucleic acid as a template (Losick & Chamberlin, 1976; von Hippel et al., 1984; McClure, 1985; Doi, 1977). These enzymes are usually large, multisubunit assemblies; *Escherichia coli* RNA polymerase contains five major subunits, with a total molecular weight of 449K. The "core" enzyme consists of subunits β' (M_r 155 162; Ovchinnikov et al., 1982) and β (M_r 150 619; Ovchinnikov et al., 1981), and two α subunits (M_r 36 512; Ovchinnikov et al., 1977); the core enzyme is capable of elongating RNA but does not specifically initiate transcription at promoter sites on DNA. The RNA polymerase holoenzyme contains the core plus the dissociable subunit σ (M_r 70 263; Burton et al., 1981), which is required for specific initiation of transcription.

Since the discovery of the σ subunit (Burgess et al., 1969) and its ability to act catalytically in the initiation of transcription (Travers & Burgess, 1969), the mechanism by which σ acts has interested many investigators (Chamberlin, 1974; Hansen & McClure, 1980; von Hippel et al., 1984; McClure, 1985). It is frequently assumed that σ dissociates from the transcription complex shortly after formation of the first phosphodiester bond (Lewin, 1983), but the elegant experiment of Hansen and McClure (1980) indicates that σ remains bound until either eight or nine bases have been transcribed on a poly[d(A-T)] template. No other σ dissociation experiments have been described in the literature, presumably because they are very difficult technically, but it has been emphasized recently that the question of when and why σ dissociates from a transcription complex is of fundamental importance for understanding the mechanism of transcription (von Hippel et al., 1984; McClure, 1985).

Photoaffinity experiments with RNA analogues in transcription complexes can provide relevant information, since if photoaffinity labeling of σ occurs, it must have been part of a transcription complex. DeRiemer and Meares (1981b) found that a 5′-azide photoprobe on a di- or trinucleotide

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