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Transcription Boundaries of U1 Small Nuclear RNA†

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Transcription-proximal stages of U1 small nuclear RNA biosynthesis were studied by ^{32}P labeling of nascent chains in isolated HeLa cell nuclei. Labeled RNA was hybridized to nitrocellulose-immobilized, single-stranded M13 DNA clones corresponding to regions within or flanking a human U1 RNA gene. Transcription of U1 RNA was inhibited by >95% by α-amanitin at 1 μg/ml, consistent with previous evidence that it is synthesized by RNA polymerase II. No hybridization to DNA immediately adjacent to the 5′ end of mature U1 RNA (−6 to −105 nucleotides) was detected, indicating that, like all studied polymerase II initiation, transcription of U1 RNA starts at or very near the cap site. However, in contrast to previously described transcription units for mRNA, in which equimolar transcription occurs for hundreds or thousands of nucleotides beyond the mature 3′ end of the mRNA, labeled U1 RNA hybridization dropped off sharply within a very short region (~60 nucleotides) immediately downstream from the 3′ end of mature U1 RNA. Also in contrast to pre-mRNA, which is assembled into ribonucleoprotein (RNP) particles while still nascent RNA chains, the U1 RNA transcribed in isolated nuclei did not form RNP complexes by the criterion of reaction with a monoclonal antibody for the small nuclear RNP Sm proteins. This suggests that, unlike pre-mRNA-RNP particle formation, U1 small nuclear RNP assembly does not occur until after the completion of transcription. These results show that, despite their common synthesis by RNA polymerase II, mRNA and U1 small nuclear RNA differ markedly both in their extents of 3′ processing and their temporal patterns of RNP assembly.

U1 RNA is one of the stable, abundant, small nuclear RNAs characteristic of most eucaryotic cells (C. Brunel, J. Sri-Widada, and P. Jeanteur, Prog. Mol. Subcell. Biol., in press). The functional form of U1 RNA is a ribonucleoprotein (RNP) complex (U1 small nuclear RNP [snRNP]) that contains at least eight proteins (34, 42) and is immunoprecipitated by antisera from patients with certain autoimmune diseases (Brunel et al., in press). Several lines of evidence now point to an involvement of U1 snRNP in mRNA splicing (2, 5, 15, 18, 21, 24, 34, 36, 39, 49).

The biosynthesis of U1 snRNP itself is a complex process that has just begun to be elucidated. Human cells contain approximately 30 U1 RNA genes per haploid genome (27), clustered on chromosome I (26). U1 RNA is synthesized by RNA polymerase II (19, 40) but, although the 5′ sequences flanking several cloned human U1 genes are highly conserved (27, 31), they lack the canonical promoter sequences characteristic of other polymerase II-transcribed genes (4). Moreover, the transcription unit of U1 RNA has not been clearly defined. UV target inactivation mapping has suggested a U1 RNA transcription unit of approximately 5,000 nucleotides (11). On the other hand, only 231 and 35 nucleotide pairs of the 5′ and 3′ flanking DNA, respectively, are necessary for the transcription of a human U1 RNA gene when injected into *Xenopus laevis* oocytes (43). RNA transcribed in the oocyte from such templates is initiated at nucleotide +1, corresponding to the mature U1 RNA cap site (3, 43). In addition, U1 RNA precursor molecules 1 to 12 nucleotides longer than mature U1 RNA have been detected in pulse-labeled HeLa cells (10, 12, 29, 51). These U1 RNA precursors are capped (47a), indicating that they are extended at their 3′ ends. Longer U1 RNA precursors, corresponding to those predicted by the earlier UV target mapping (11), have not been found (G. R. Kunkel, E. D. Wieben, and T. Pederson, unpublished observations).

The available information thus predicts a U1 RNA transcription unit bounded at the 5′ end by the cap site and ending perhaps only 15 to 35 nucleotides beyond the mature 3′ end of U1 RNA. In the present investigation, we used run-on labeling in isolated HeLa cell nuclei to examine the U1 RNA transcription unit. Our data confirm the predicted transcription boundaries and illuminate features by which U1 RNA biosynthesis appears to differ from that of mRNA.

(Brief accounts of this work were presented at the 1984 American Society for Cell Biology annual meeting and the 1985 University of California, Los Angeles Symposium on Nuclear Envelope Structure and RNA Maturation.)

MATERIALS AND METHODS

Cells. HeLa cells were maintained in suspension culture as described previously (37). All experiments were performed with nuclei harvested from exponentially growing cells. For some experiments cells were labeled for 20 to 22 h with 10 μCi of [5-^{3}H]uridine per ml.

Nuclear isolation. All procedures were carried out on ice unless otherwise noted. Cells were broken by homogenization in hypotonic buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris [pH 7.4]) without detergent. Nuclei were isolated by centrifugation through a 10-ml cushion of 2 M sucrose in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5)–5 mM magnesium acetate–0.1 mM EDTA (16,500 rpm for 45 min in a Beckman SW27 rotor) and then suspended in 25% glycerol–50 mM HEPES (pH 7.5)–5 mM magnesium acetate–0.1 mM EDTA–5 mM β-mercaptoethanol. Portions of 30 μl, each containing 3×10^6 nuclei, were individually stored at −80°C for up to 6 months.

Transcription in vitro and RNA isolation. Transcription reactions were typically carried out in a final volume of 100 μl containing 30 μl of nuclei, 7.5% glycerol, 19 mM HEPES (pH 7.5), 3.8 mM magnesium acetate, 0.5 mM $MnCl_2$, 84 mM

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potassium acetate, 25 mM ammonium acetate, 30 μM EDTA, 50 μM EGTA, 3 mM β-mercaptoethanol, 130 μg of creatine phosphokinase per ml, 8 mM creatine phosphate, 0.5 mM *S*-adenosylmethionine, 1 mM ATP, 0.25 mM CTP, 0.25 mM UTP, 40 μM cold GTP, and 100 μCi of [α-^{32}P]GTP (760 Ci/mmol; New England Nuclear Corp.). Samples were incubated at 25°C for 30 min. NaCl and DNase I were then added to 0.6 M and 60 μg/ml, respectively, and incubation was continued at 25°C for 10 min. Total RNA was isolated by phenol-chloroform extraction in 1% sodium dodecyl sulfate–4 M urea, followed by ethanol precipitation. Unincorporated ribonucleoside triphosphates were removed by chromatography of RNA samples on Sephadex G-50.

In some experiments nuclear transcriptions were run in high salt-heparin-Sarkosyl. These reactions were formulated and run as described above, except that the incubation buffer contained 0.35 M ammonium sulfate, 1 mg of heparin per ml, 0.6% Sarkosyl, and no potassium acetate or ammonium acetate (22).

Transcription of U1 RNA from an SP6 promoter. The desired U1 DNA template was prepared by subcloning the *Bgl*II-*Eco*RI fragment from plasmid pD2 (31) into *Bam*HI-*Eco*RI doubly cut pSP62-PL DNA. This SP6 promoter-U1 DNA construct (SP6/U1) was kindly provided by Eric Wieben, Department of Cell Biology, The Mayo Foundation, Rochester, Minn. (see also Fig. 1 and 4A). *Eco*RI-linearized DNA was transcribed by SP6 RNA polymerase (New England Nuclear Corp.), with [α-^{32}P]GTP used as a tracer (32). After DNase treatment (32), the RNA transcripts were purified by phenol-chloroform extraction and Sephadex G-50 chromatography.

Subcloning of recombinant M13 DNAs. Regions of DNA from pD2, a plasmid containing a human U1 RNA gene (31), were subcloned into the M13 bacteriophage vectors mp8 and mp9 (33). Figure 1 shows the regions included in each recombinant phage. Subclones A, B, C, and F were constructed by ligating the desired gel-purified restriction fragment into the replicative form of mp8 or mp9 DNA (cleaved with the appropriate restriction endonuclease) and transfected into competent *Escherichia coli* JM103 (33). *Dde*I termini were made blunt-ended by filling with the Klenow fragment of DNA polymerase I and ligated into the *Sma*I site of an M13 vector. Subclone E was constructed by digestion of the ~600-base-pair *Taq*I-*Eco*RI fragment from pD2 with BAL 31 nuclease, end-filling with the Klenow fragment of DNA polymerase I, and ligation into M13 mp9 replicative form DNA which had been linearized with *Sma*I and dephosphorylated by treatment with calf intestinal alkaline phosphatase.

For the construction of subclone D, a synthetic oligodeoxynucleotide with the sequence corresponding to the first 15 nucleotides downstream of the mature 3′ end of U1 RNA, ACTTTCTGGAGTTTC (noncoding strand), was annealed to single-stranded DNA of M13 subclone A and used to prime the synthesis of a long stretch of downstream U1 DNA by the Klenow fragment of DNA polymerase I. This partially double-stranded DNA was restricted by *Dde*I endonuclease and treated with S1 nuclease to remove all single-stranded regions. A diffuse band corresponding to ~70 base pairs in size was gel purified, ligated into M13 mp9 replicative form DNA which had been linearized with *Sma*I and dephosphorylated with calf intestinal alkaline phosphatase, and transfected into competent *E. coli* JM103.

Slot hybridization of nuclear RNA. M13 DNAs were immobilized on nitrocellulose (2 μg per slot) with a Minifold II apparatus (Schleicher & Schuell, Inc.). The sheets were washed with 4× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate), baked at 80°C for 2 h, and prehybridized for 4 to 24 h at 42°C with 50% formamide–5× SSC–50 mM sodium phosphate (pH 7.0)–0.1% sodium dodecyl sulfate–1 mg of yeast tRNA per ml–0.1% each bovine serum albumin, Ficoll, and polyvinylpyrrolidone. Immediately before hybridization, nuclear RNA samples were heated at 80°C for 5 min. DNA-RNA hybridization took place for 42 to 48 h at 42°C in 50% formamide–5× SSC–50 mM sodium phosphate (pH 7.0)–0.1% sodium dodecyl sulfate–0.04% each bovine serum albumin, Ficoll, and polyvinylpyrrolidone. Blots were washed in 1× SSC at room temperature, treated with 0.4 μg of RNase A per ml plus 10 U of RNase T1 per ml for 1 h at 37°C, and finally washed in 1× SSC–0.1% sodium dodecyl sulfate at 20 to 23°C. Preflashed X-ray film was exposed between two intensifying screens for up to 14 days. Autoradiographs were scanned with a Helena Quick Scan densitometer.

Immunoprecipitation. Immediately after in vitro transcription, nuclear extracts were prepared at 0 to 4°C as described previously (48). A total of 200 μg of mouse Sm monoclonal antibody (23), recovered as purified immunoglobulin G from ascitic tumor fluid (14), was added to nuclear extracts and incubated at 0°C for 1 h. Antigen-antibody complexes were recovered with Pansorbin (Calbiochem-Behring), washed with NET-2 (NaCl-EDTA-Tris), and eluted as described previously (20). RNA was isolated by phenol-chloroform extraction and ethanol precipitation and used for slot hybridization. [^{3}H]RNA from mock-transcribed nuclear samples (see below) was fractionated on 10% acrylamide–8.3 M urea gels as described previously (48).

RESULTS

Mapping U1 RNA transcription in isolated nuclei. A cloned human U1 RNA gene (pD2 [31]) was subcloned in M13 mp8 and mp9 vectors to produce DNA hybridization probes adjacent to and within the U1 RNA-colinear region (see above and Fig. 1). After immobilization on nitrocellulose, these DNAs were hybridized with ^{32}P-labeled RNA generated by transcription in isolated nuclei. The DNA-RNA hybrids were digested with RNase so that ^{32}P radioactivity could be related to the mass of RNA hybridized to a DNA fragment of known length. Typical results are shown in Fig. 2. It can be seen that hybridization to the DNA immediately upstream from the U1 RNA-colinear region (clone B, Fig. 1) was not appreciably above background (compare with mp9 DNA control slots). This indicates either that transcripts from this region are rapidly degraded during the nuclear incubation or, more likely (43), that U1 RNA transcription starts at or very near the cap site. An almost complete suppression of U1 RNA labeling was observed when in vitro transcription was carried out in the presence of α-amanitin at a concentration of 1 μg/ml (Fig. 2), consistent with previous evidence that U1 RNA is transcribed by RNA polymerase II (19, 40). The most interesting result in Fig. 2, however, is the greatly reduced level of RNA hybridizing to DNA downstream from the mature 3′ end of U1 RNA (compare slots of fragments D or E with those of fragment C). We shall return presently to a quantitative analysis of these results.

Since large amounts (~10^6 molecules per nucleus) of mature, unlabeled U1 RNA were present in the ^{32}P-labeled RNA samples, it was essential to verify that sufficient DNA sequence excess prevailed in the hybridization reactions to provide a linear response between ^{32}P signal and amount of labeled U1 RNA. We found that the level of hybridization of

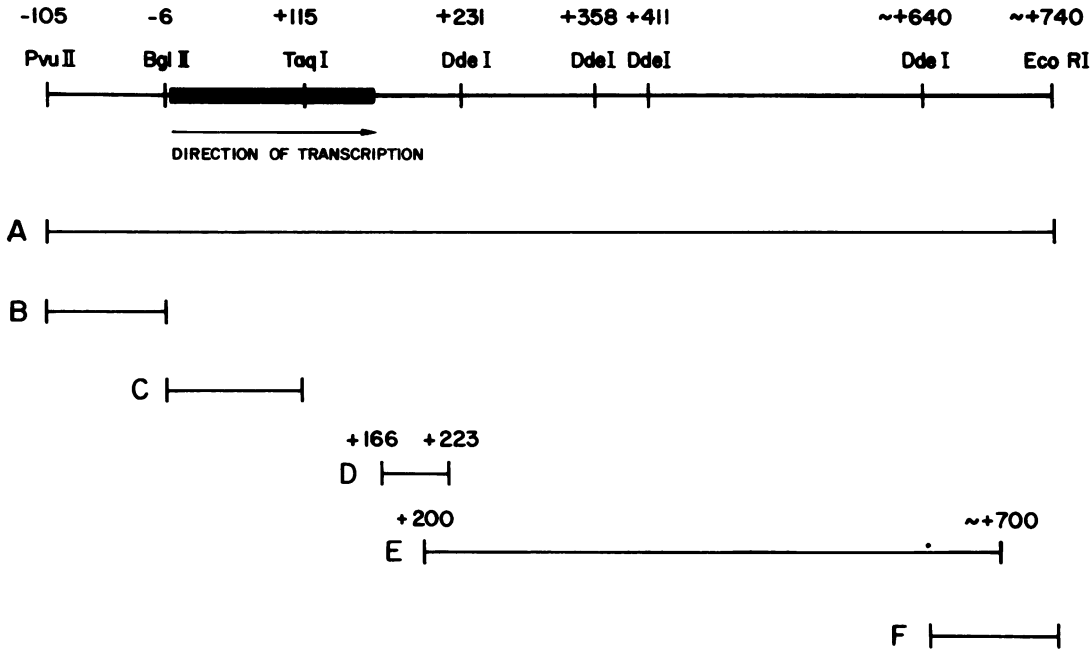

FIG. 1. Human U1 DNA and M13 subclones. DNA used for M13 subcloning was restricted from plasmid pD2, a human U1 RNA gene isolated by Manser and Gesteland (31). The black box indicates DNA sequences complementary to mature U1 RNA (+1 to +164). DNA fragments were subcloned into the appropriate vector (either M13 mp8 or mp9) and are denoted by letters A through F.

labeled RNA to the downstream fragment E relative to that of the mature U1 RNA-colinear fragment C was the same irrespective of whether the mature U1 RNA was first removed by an immunoprecipitation procedure that excluded the U1 RNA labeled in vitro (see Fig. 6). In other control experiments, we found that the observed amount of U1 RNA hybridization to identically loaded slot blots was directly proportional to the dilution of total labeled nuclear RNA (data not shown).

The low level of RNA hybridization observed with DNA fragments D and E (Fig. 2) could represent elongated U1 RNA transcripts or, alternatively, new initiation events, the latter reflecting promiscuous polymerase binding or the presence of another authentic transcription unit lying 3′ to the U1 gene. To examine this point, we first hybrid-selected ^{32}P-labeled U1 RNA from nuclear transcription reactions, using DNA clone C (Fig. 1). These hybridized, U1 RNA-containing molecules were then eluted from the DNA and rehybridized to downstream clones D through F (Fig. 3). The levels of hybridization to clones D and E relative to C were the same as was observed with total RNA (compare Fig. 3 with Fig. 2). This observation indicates that the hybridization to clones D and E represents RNA molecules that contain covalently linked U1 sequences. Thus, although most U1 RNA chains labeled in nuclei ended near the mature RNA 3′ terminus, a small fraction of these transcripts were elongated several hundred nucleotides beyond this point.

Quantitation of hybridization downstream of the U1 RNA mature 3′ end. To quantitate the slot hybridization signals, we prepared a U1 RNA transcript containing sequences far into the 3′ flanking region. An appropriate DNA template was constructed by ligating the *Bgl*II-*Eco*RI fragment from

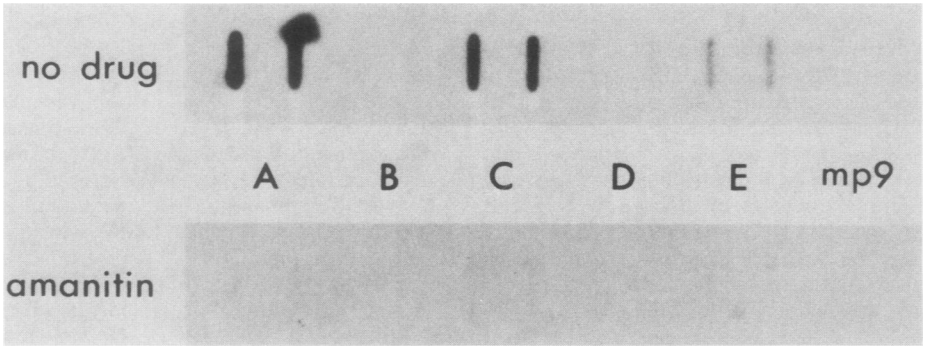

FIG. 2. Transcription analysis of U1 RNA. Nuclei were incubated as described in the text in the presence of [α-^{32}P]GTP with or without 1 μg of α-amanitin per ml. Total RNA was isolated from both samples and hybridized to M13 DNAs immobilized on duplicate slot blots. Nitrocellulose sheets were washed, treated with RNases A and T1, and exposed to preflashed X-ray film with intensifying screens (see text). A, B, C, D, and E correspond to the M13 subclones diagrammed in Fig. 1. Control M13 DNA containing no insert of human DNA was immobilized in the slots designated mp9.

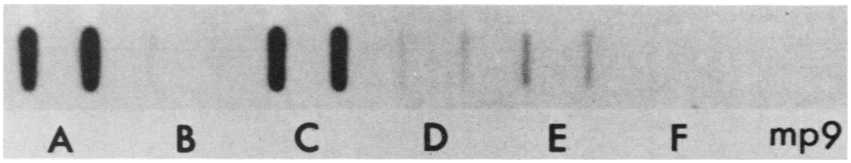

FIG. 3. Downstream RNA transcribed in vitro is part of an RNA chain containing mature U1 sequences. RNA was isolated from nuclei after in vitro transcription and selected by hybridization to 25 μg of DNA from M13 subclone C, which was immobilized on a small nitrocellulose filter circle (28). Hybrid-selected RNA was eluted, mixed with 50 μg of tRNA, precipitated with ethanol, and hybridized to slot blot-immobilized M13 DNAs. Hybrids were digested with RNase as described in the legend to Fig. 2.

the human U1 DNA clone pD2 adjacent to the *Salmonella* phage SP6 promoter (Fig. 4A). Transcription of this DNA with SP6 RNA polymerase generated a U1 RNA molecule with a long trailer sequence extending approximately 600 nucleotides beyond the U1 mature 3′ end (Fig. 4A). Hybridization of this transcript to the various DNA clones, followed by RNase digestion, provided a standard for full-length RNA hybrid on each DNA fragment tested. This, then, constitutes a model case for uninterrupted, stoichiometric transcription downstream beyond the U1 mature 3′ end, against which the hybridization signals of RNA from nuclear run-on transcription can be quantitated. Hybridization results for the SP6-transcribed elongated U1 RNA are shown in Fig. 4B, along with comparable data for RNA from nuclear run-on transcription. By integration of densitometric scans (Fig. 4C), the relative amounts of downstream DNA hybridization were then calculated and plotted against that of fragment C (Fig. 5; see legend for details). It can be seen that the RNA from nuclear run-on transcription is vastly underrepresented in the downstream DNA region. The relative hybridization, corrected for DNA fragment lengths (see Fig. 5 legend), to downstream DNA (clones D through F) was 8 to 14% of that of the internal U1 RNA sequences (clone C). Together with the results in Fig. 3, these data indicate that transcription stops near the mature 3′ end of U1 RNA in 86 to 92% of the cases.

We are reasonably confident that the dearth of downstream transcription does not reflect rapid 3′ processing in the isolated nuclei. First, we found no change in the RNA hybridization ratio of clones E to C when nuclei were chased for 2 h with a 100-fold excess of unlabeled GTP (data not shown). In addition, a short (5 min) nuclear transcription reaction revealed no enrichment of downstream hybridization relative to that seen with the usual 30-min transcription period (data not shown).

High sequence congruence of hybridized RNA and the downstream DNA probes used. Since there are several human U1 genes, it is possible that the relative lack of downstream hybridization reflects divergence of sequences 3′ to the human U1 gene in clone pD2 on the one hand (clones D through F) and those 3′ of the one or more U1 genes being transcribed in HeLa cell nuclei on the other. Although sequences immediately 3′ of several human U1 genes are highly conserved (27, 31), clones E and F cover a much longer expanse of 3′ DNA. This, therefore, is not a trivial possibility. To examine this point, we carried out nuclear run-on transcriptions in the presence of high salt-heparin-Sarkosyl. As previously described for ovalbumin mRNA transcription in isolated chicken oviduct nuclei (22), these conditions permit RNA elongation beyond normal termination sites, presumably owing to induced alterations of chromatin structure in these regions. A representative slot blot hybridization analysis of such labeled RNA is shown in Fig. 4B, with the corresponding densitometric scan shown in Fig. 4C. It can be seen that RNA transcribed under these conditions hybridized to downstream regions in amounts very similar to the model case of stoichiometric transcription towards the 3′ end (SP6/U1 in Fig. 4C; see also Fig. 5). This demonstrates that the particular human U1 gene clone we used has the same or a very similar 3′ flanking sequence as the transcriptionally active HeLa U1 gene(s). This, then, is a positive control that provides further confidence in the relative lack of downstream hybridization observed with RNA from nuclear transcription performed under normal conditions.

RNP status of newly transcribed U1 RNA. mRNA transcripts are known to be packaged into RNP particles while still nascent RNA chains, as shown both by electron microscopy of spread chromatin (1) and by RNA-protein cross-linking of cells labeled extremely briefly with [^{3}H]uridine (9). In contrast, the stage of U1 RNA biosynthesis at which RNP assembly takes place is not known. We therefore investigated whether U1 RNA transcribed in isolated nuclei is assembled into RNP by the criterion of its reaction with a monoclonal antibody for the Sm proteins of snRNPs (23). The efficacy of the antibody reaction was monitored by using nuclei from cells that had been prelabeled in vivo with [^{3}H]uridine. After in vitro transcription in the presence or absence of [α-^{32}P]GTP, parallel immunoprecipitations were carried out. Analysis of [^{3}H]RNAs by gel electrophoresis and fluorography (Fig. 6A) revealed that the antibody efficiently selected the U1 snRNP (as well as those containing U2, U4, U5, and U6 RNAs, as expected). In contrast, slot hybridization analysis of the ^{32}P-labeled U1 RNA transcribed in nuclei in vitro revealed that the great majority was not in an RNP form reactive with the Sm antibody (Fig. 6B). This indicates that, at least under the conditions of nuclear transcription, newly synthesized U1 RNA is not assembled into the characteristic snRNP complex.

DISCUSSION

The U1 RNA primary transcript. The data we have presented demonstrate that, in isolated HeLa cell nuclei, U1 RNA transcription does not proceed far beyond the mature 3′ end of this RNA in the majority of cases. Since very little hybridization was observed even with DNA clone D, representing 57 nucleotides immediately beyond the 3′ end of mature U1 RNA (Fig. 1), it follows that transcription stops either at nucleotide +164 or somewhere within the region between +165 and +223. We have been unable to dissect this region further because the required subsegments of clone D would be so small as to invite hybrid instability. The previous demonstration in vivo of capped U1 RNA precursors elongated 1 to 12 nucleotides (29, 47a), however, strongly suggests that the 3′ boundary of U1 transcription lies beyond nucleotide +164. Thus, our present results are in accord with the in vivo data and constitute one of

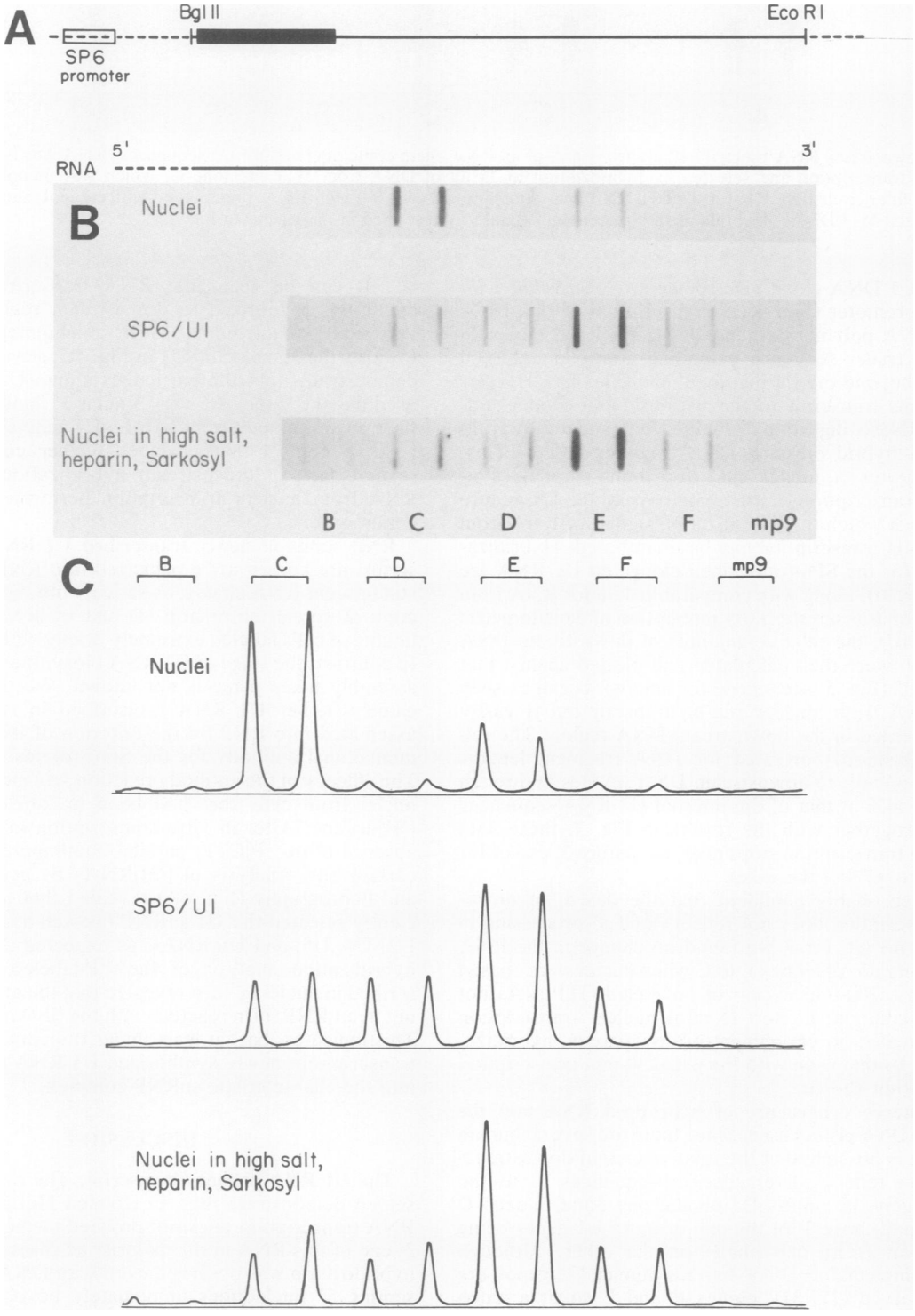

FIG. 4. Hybridization pattern of a 3'-elongated artificial U1 RNA as a model case for stoichiometric downstream transcription. (A) SP6/U1 construction and run-off transcript. Human DNA contained in the *Bgl*II-*Eco*RI fragment from plasmid pD2 was subcloned into *Bam*HI-*Eco*RI-treated pSP62 DNA. The thick black box indicates sequences corresponding to mature U1 RNA. Transcription of *Eco*RI-linearized pSP62/U1 DNA by SP6 RNA polymerase yielded a model U1 RNA containing a stoichiometric level of downstream sequences. – – – – –, pSP62 sequences; ———, pD2 (U1) sequences. (B) Comparison of slot blots hybridized against RNA from nuclei incubated under standard in vitro transcription conditions (top), ~800 nucleotides. SP6/U1 synthetic RNA (middle), and RNA transcribed from isolated nuclei incubated in 0.35 M $(NH_4)_2SO_4$–1 mg of heparin per ml–0.6% Sarkosyl (bottom). (C) Densitometric scans of slot blots from panel B. Units on the vertical axis are arbitrary, and absolute peak heights from different scans should not be compared.

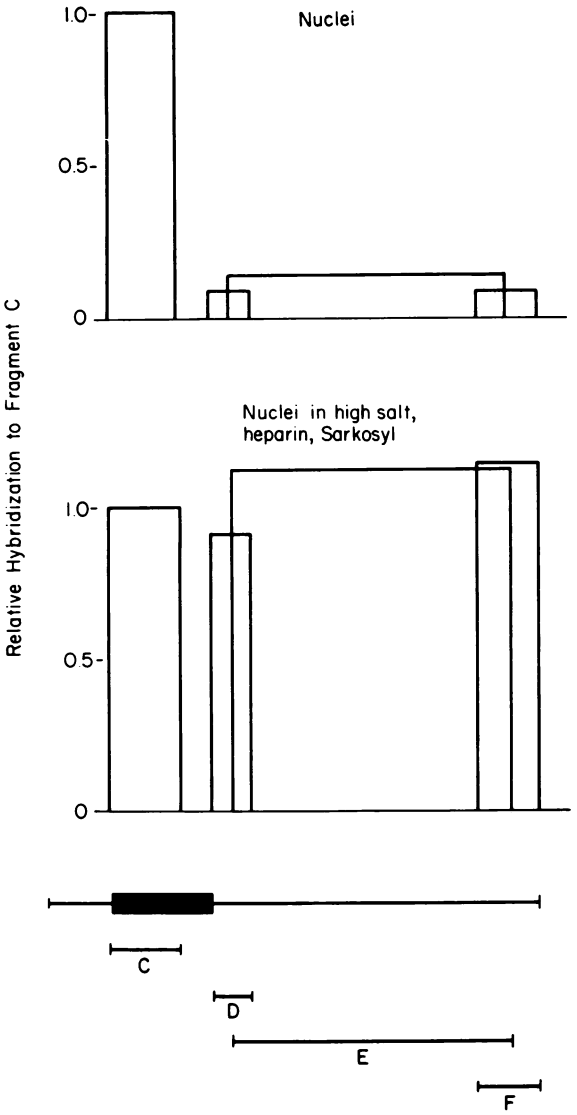

FIG. 5. Quantitation of in vitro nuclear transcription downstream from the mature 3′ end of U1 RNA. Densitometric scans from Fig. 4C were integrated and used to compute ratios of hybridization for each downstream region (D, E, or F) to coding region C for each experiment. The relative ratio $(X/C)_{nuclei}/(X/C)_{SP6/U1}$ describes the fraction of ^{32}P-labeled RNA present for a downstream region (X) relative to coding region C, normalized for the number of G nucleotides in each region. (A) Relative hybridization when nuclei were incubated in vitro under standard transcription conditions (see text). (B) Results from in vitro transcription carried out in the presence of high salt-heparin-Sarkosyl. Aligned at the bottom of the figure is a schematic drawing from Fig. 1 of the relative positions of each DNA region used for this analysis.

four cases now recorded (6, 16, 17) in which transcription unit mapping results from isolated nuclei are in close agreement with data on in vivo precursor RNAs.

What, then, is to be made of the low level of hybridization seen in the downstream region of the U1 gene? The trivial possibilities that this reflects random polymerase binding or the activity of another transcription unit altogether are ruled out by the demonstration that the RNA hybridizing to these downstream clones contain covalently linked mature U1 RNA sequences (Fig. 3). We therefore favor a probabilistic model in which there is a high likelihood of U1 RNA termination within 57 nucleotides of the mature 3′ end but with polymerase passing beyond this region in about 10% of the cases. Whether the more frequent termination involves a specific site of one or more nucleotides or is random over the entire high-probability zone (nucleotides +165 to +223) cannot be ascertained from our data. A putative polymerase II termination sequence has been found 3′ of a rat U2 RNA gene (44), at a location wholly consistent with the sizes of U2 RNA precursor molecules detected in vivo (44, 47a).

Our finding that there is no appreciable hybridization to 5′ flanking DNA is in complete accord with the recent demonstration in *Xenopus* oocytes that human U1 RNA transcription initiates at the cap site (43) and not further upstream, as was initially reported for U1 DNA transcribed in a whole-cell extract (35). Thus, U1 small nuclear RNA conforms to the rule, presently without exceptions, that all RNA polymerase II transcripts initiate at nucleotide +1 of the mature RNA.

Our data also bear on previous size estimates of U1 RNA transcription units by UV target inactivation kinetics (11, 13). The first of these studies indicated a U1 transcription unit some 4,000 to 5,000 nucleotides in length (11). Our data do not support such a large U1 RNA transcription unit and, as indicated above, attempts to find U1 RNA molecules this large in vivo by several approaches have not been successful. A recent study (13) raises the possibility that the UV hypersensitivity of U1 RNA synthesis may be due to factors other than DNA target size per se.

U1 RNA transcription and snRNP assembly may not be temporally coupled. U1 RNA synthesized in isolated nuclei does not become assembled into RNP complexes, by the criterion of reactivity with a monoclonal antibody against characteristic snRNP proteins (Fig. 6). It is possible that this merely reflects an insufficient pool of these proteins in isolated nuclei or other conditions that are nonpermissive for snRNP assembly. It is of interest, however, that pre-mRNA produced in the same isolated nuclear transcription system does assemble into RNP complexes characteristic of that RNA class (8), an observation that argues against wholesale leakage of free RNP proteins during nuclear isolation.

The snRNP proteins that react with Sm antibody bind to a region near the 3′ end of (mature) U1 RNA (25, 38). If the array of U1 RNA molecules that become labeled in isolated nuclei were all still incomplete at the end of the in vitro transcription reaction, then only a small portion of these nascent RNA chains would contain binding sites for the Sm antibody-reactive snRNP proteins. This scenario is unlikely on the basis of the following considerations. The rates of RNA polymerase II elongation in isolated nuclei under conditions very similar to ours have been estimated to be between 10 and 500 nucleotides per minute (30, 41, 45). We therefore infer that, in our standard reactions of 30 min, each polymerase would move at least 300 nucleotides. It is therefore very likely that the great majority of labeled U1 RNA molecules in our experiments are completed transcripts and possess snRNP protein binding sites.

Our results therefore raise the possibility that U1 RNA transcription and snRNP assembly are not temporally coupled in the cell, which receives indirect support from several recent observations. *Xenopus* oocytes contain cytoplasmic stores of snRNP proteins, showing that these are not, by themselves, karyophilic proteins (50). Injection of human U1 RNA into enucleated *Xenopus* oocytes results in snRNP formation (7), showing that this process can occur in the cytoplasm. Moreover, U1 and U2 RNAs assemble into

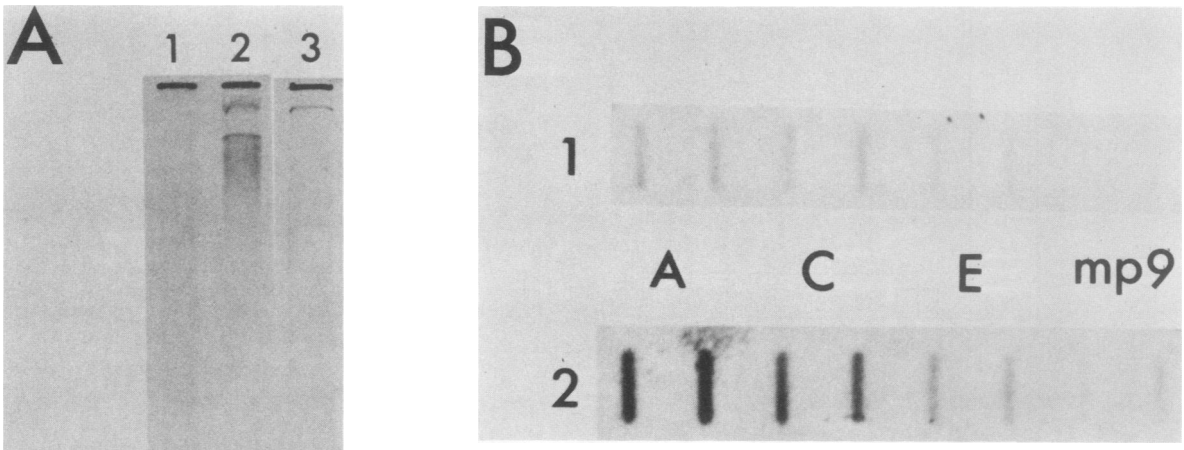

FIG. 6. Most U1 RNA transcribed in isolated nuclei is not bound to Sm antigen. Nuclei from HeLa cells labeled overnight with [^{3}H]uridine were incubated in the absence (A) or presence (B) of [α-^{32}P]GTP. Nuclear lysates were immunoprecipitated with monoclonal Sm antibody, and RNA was isolated as described in the text. (A) Gel electrophoresis of [^{3}H]RNAs from mature snRNPs in 10% polyacrylamide containing 8.3 M urea. RNA loaded in each lane of the gel was from an equivalent volume of nuclear lysate. Lane 1, no antibody; lane 2, Sm antibody, Pansorbin-bound fraction; lane 3, Sm antibody, nonbound fraction. (B) Hybridization analysis of ^{32}P-labeled U1 RNA. RNA samples were hybridized to DNAs on identically loaded blots as described in the text. Blot 1, Sm antibody, Pansorbin-bound fraction; blot 2, Sm antibody, nonbound fraction.

snRNPs when added to a rabbit reticulocyte lysate (46, 47), which is, again, a cytoplasmic fraction. In addition, several studies (10, 12, 28, 29, 47a, 51) have revealed that mammalian pulse-labeled U1, U2, and U4 RNA precursors appear promptly in cytoplasmic fractions (defined strictly operationally by various homogenization procedures). These cytoplasmic precursors are found in snRNPs, whereas snRNP particles containing small nuclear RNA precursors cannot be found in the corresponding nuclear fractions (28, 29, 47a). Taken together, these previous observations and the present results suggest that snRNP assembly takes place well after completion of U1 RNA transcription. This delayed snRNP assembly of small nuclear RNAs, as opposed to the cotranscriptional RNP assembly of pre-mRNA (1, 9), appears to be yet another difference in the overall biosynthesis of these two classes of RNA polymerase II transcripts.

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