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Transcription initiation by RNA polymerase II is inhibited by *S*-adenosylhomocysteine

(*in vitro* transcription/cap formation/initiation complex)

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ABSTRACT **Most eukaryotic mRNAs are blocked at their 5′ termini by guanylylation and methylation. These "cap structures" have been shown to play important roles in increasing the stability and translatability of mRNAs. Previous *in vitro* and *in vivo* data suggest that these modifications occur extremely early in the synthesis of RNA transcripts by RNA polymerase II. Here we show that *S*-adenosylhomocysteine (AdoHcy), both a product and an inhibitor of transmethylation reactions, inhibits transcription initiation by RNA polymerase II, but not by RNA polymerase III, in a HeLa whole-cell lysate. AdoHcy must be present during initiation to inhibit transcription and does not affect elongation by RNA polymerase II or the stability of the resultant transcript. Furthermore, AdoHcy does not inhibit transcription by purified HeLa RNA polymerase II. These results suggest that formation of the 5′-cap structure is coupled to initiation of transcription and is consistent with a close association between the capping enzymes and RNA polymerase II at the time of initiation.**

Eukaryotic mRNAs transcribed by endogenous cellular RNA polymerase II or by virion-associated RNA polymerases are almost without exception modified at the 5′ terminus by a cap structure of the general form $m^7G(5')pppN$- (see ref. 1 for review). The basic cap structure (cap 0) can be further methylated at the 5′-terminal penultimate residue [cap I, $m^7G(5')pppNm$-] and its adjacent residue [cap II, $m^7G(5')pppNm$-Nm-] in higher eukaryotes and their viruses (1). These cap structures have been shown to increase the stability and translatability of mRNAs and thus play important roles in the metabolism and utilization of mRNAs (2–4). Although many details of the mechanism of cap formation have been elucidated from *in vitro* studies using virion-associated RNA polymerases (1), much less is known about the molecular events involved in formation of the corresponding structures in cellular mRNAs.

Most of the enzymes that catalyze the synthesis of cap structures of the type $m^7G(5')pppNm$(-Nm)- in HeLa cells have been identified and at least partially purified and characterized (5–9). The reactions involved in cap formation as deduced by these studies are

(*i*) pppN-N- → ppN-N- + P_i
(*ii*) GTP + ppN-N- → $G(5')pppN$-N- + PP_i
(*iii*) AdoMet + $G(5')pppN$-N- → $m^7G(5')pppN$-N- + AdoHcy
(*iv*) AdoMet + $m^7G(5')pppN$-N- → $m^7G(5')pppNm$-N- + AdoHcy
(*v*) AdoMet + $m^7G(5')pppNm$-N- → $m^7G(5')pppNm$-Nm- + AdoHcy,

where pppN-N- represents the 5′ end of the nascent RNA chain and AdoMet and AdoHcy are *S*-adenosylmethionine and *S*-adenosylhomocysteine, respectively.

Reactions *i–iv* are catalyzed by nuclear enzymes, while reaction *v* probably occurs in the cytoplasm (9). Previous *in vitro* transcription experiments using nuclei isolated from adenovirus serotype 2 (Ad2)-infected HeLa cells suggested that cap formation is linked with initiation of transcription by RNA polymerase II (10), consistent with the finding that the cap site and the initiating residue of Ad2 primary transcripts appear to be coincident (11). The development of soluble mammalian *in vitro* transcription systems (12, 13) capable of specific initiation by RNA polymerase II on defined exogenously added DNA templates has opened the way for examining the molecular details of the dynamic processes involved in the biosynthesis of cellular mRNA. In addition to accurate transcription initiation, the HeLa whole-cell extract has been shown to modify the 5′ termini of newly synthesized transcripts by guanylylation and methylation (13).

In the cytoplasmic polyhedrosis virus system, cap formation appears to be coupled with initiation of transcription, and it has been suggested that the mechanism of this coupling involves a close association between the virus RNA polymerase and capping enzymes (14). To investigate further the molecular mechanisms of cap formation and transcription initiation by cellular enzymes, *in vitro* transcription experiments were carried out in the presence of AdoHcy, an inhibitor of transmethylation reactions. Addition of AdoHcy to a soluble whole-cell extract made from HeLa cells prevents the appearance of "run-off" transcripts by RNA polymerase II but has no effect on transcription by RNA polymerase III. The effect on RNA polymerase II transcription is shown to occur at initiation and not be due to inhibition of elongation or increased instability of the RNA. These results suggest that the 5′-modification enzymes are components of the initiation complex.

MATERIALS AND METHODS

Preparation of Recombinant DNA. Adenovirus serotype 2 DNA was isolated from purified virions grown in HeLa cells (15) and the *Hin*dIII-B restriction endonuclease fragment was cloned in pBR322 as described (16). The recombinant pH3B*R was constructed by inserting the *Hin*dIII-B fragment into the clone pXB210 containing the major late promoter of Ad2 (16). The Ad2 recombinant pϕ4 has already been described (16). T. Maniatis provided two recombinants containing the human β-globin gene cloned from genomic DNA. The first is a 7.5-kilobase (kb) *Hin*dIII restriction fragment cloned in pBR322. The second, which includes *Alu* I repetitive sequences flanking the β-globin gene, is a λ clone containing a 9.3-kb *Bam*HI restriction fragment, which was subcloned in pBR322 by M. Colozzo. Purified pVAtk10 and simian virus 40 (SV40) DNAs were the

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Abbreviations: AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; Ad2, adenovirus serotype 2; m.u., map unit(s); SV40, simian virus 40; kb, kilobase(s); VA, virus-associated RNA.

gifts of D. Lewis and G. Blanck, respectively. Plasmid DNA was amplified and purified by standard protocols (17).

***In Vitro* Transcription and Analysis of RNA.** HeLa whole-cell extracts were prepared essentially as described (13). Standard 25-μl reaction mixtures were 24 mM Tris·HCl, pH 7.9/6 mM $MgCl_2$/60 mM KCl/0.06 mM EDTA/1.2 mM dithiothreitol/10% glycerol/50 μM each ATP, CTP, UTP, GTP containing 5–10 μCi of [α-^{32}P]GTP (New England Nuclear; 1 Ci = 3.7×10^{10} becquerels)/4 mM creatine phosphate, containing 15 μl of extract and 1.25 μg of DNA. α-Amanitin (Sigma), AdoMet, and AdoHcy (Calbiochem or Boehringer Mannheim) were added to reaction mixtures as indicated. AdoMet and AdoHcy (20 mM solutions) were either prepared in 4 M acetic acid and repeatedly lyophilized before use or prepared in 20 mM hydrochloric acid and used directly in transcription reactions.

Reaction mixtures were incubated at 30°C for the times indicated, and RNA was purified as described (13). Purified RNA was glyoxalated and one-quarter of each sample was analyzed by agarose gel electrophoresis according to the method of McMaster and Carmichael (18) followed by autoradiography of dried gels by exposure to Kodak XAR-5 x-ray film without intensifying screens. Exposures at room temperature were generally between 12 and 24 hr. Autoradiograms were scanned with a Gilford model 250 spectrophotometer. Purified HeLa RNA polymerase II was the gift of A. Fire and M. Samuels.

Preparation of Uncapped RNA. Uncapped RNA was prepared by two approaches. In the first method, purified capped RNA transcribed *in vitro* from pH3B*R was hybridized to excess Ad2 *Hin*dIII fragment B DNA [17.0–31.7 map units (m.u.)] as described by Berk and Sharp (19, 20) with slight modifications (21). One-half of the RNA·DNA hybrids was digested with nuclease S1 at 2×10^3 units/ml (Boehringer Mannheim), and the other half was digested with RNase T1 at 125 ng/ml, RNase T2 at 1.25 units/ml, and pancreatic RNase at 125 ng/ml (Calbiochem). Prior to incubation in extracts, RNA·DNA hybrids were denatured, diluted, digested with DNase I (Boehringer Mannheim, RNase free), extracted with phenol/chloroform and with chloroform, and ethanol precipitated.

Alternatively, SV40 complementary RNA was prepared by *in vitro* transcription of *Bam*HI-cleaved SV40 DNA using purified *Escherichia coli* RNA polymerase essentially as described (22).

RESULTS

AdoHcy Prevents the Accumulation of a RNA Polymerase II Transcript *in Vitro*. The 5′ termini of *in vivo* Ad2 late transcripts are modified to cap I (m^7GpppAmpCp) and cap II (m^7GpppAmpCmp) structures (23, 24) in which the 5′-penultimate residue is encoded at 16.45 m.u. (11). It has previously been shown that RNAs transcribed by RNA polymerase II *in vitro* by using a HeLa whole-cell extract are initiated at the *in vivo* major late cap site and contain predominantly cap I structures at the 5′ termini (13, 16). To investigate the relationship between cap methylation and transcription by RNA polymerase II, the Ad2 recombinant pH3B*R (Fig. 1) was transcribed in the HeLa whole-cell extract in the presence of various concentrations of AdoHcy or AdoMet (Fig. 2). Clone pH3B*R contains the Ad2 major late promoter and cap site and the virus-associated RNA genes I and II (VA I and II) at approximately 29 m.u. (25).

pH3B*R cleaved with *Kpn* I gives a RNA polymerase II run-off transcript of the expected size, 2.5 kilobases, and VA transcripts approximately 160 nucleotides long (predominantly VA I) transcribed by RNA polymerase III (26) (Fig. 2, lane 6). RNA polymerases II and III were shown to be responsible for transcription from their respective promoters by α-amanitin sensitivity (data not shown). Concentrations of AdoHcy greater than 100 μM prevent accumulation of the 2.5 kb run-off, and the addition of AdoHcy to 1 mM results in <10% the amount of specific transcript present in the control (Fig. 2, lanes 6–12). In contrast, AdoHcy does not prevent the accumulation of stable transcripts by RNA polymerase III from the VA promoters.

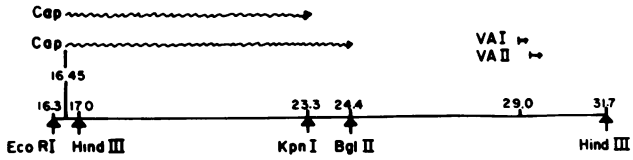

FIG. 1. Ad2 recombinant pH3B*R. The Ad2 coordinates are shown in map units (1 m.u. = 360 base pairs) above the line, and relevant restriction endonuclease cleavage sites are below the line. The wavy lines represent the RNA polymerase II run-off transcripts from *Kpn* I/*Bgl* II-cleaved pH3B*R DNA templates and the VA RNAs transcribed by RNA polymerase III. The polymerase II transcripts originate from the major late cap site at 16.45 m.u., and the VA RNAs are encoded at approximately 29.0 m.u.

At 1 mM AdoHcy, there appeared to be a reproducible enhancement of polymerase III transcription (Fig. 2, lane 12). This was found to be due to the acid used to solubilize the AdoHcy; an equimolar amount of HCl without AdoHcy had the same effect on polymerase III transcription but had no effect at all on polymerase II transcription. This acid-generated effect on polymerase III could be abolished by repeated lyophilization of AdoHcy before use (data not shown).

Addition of AdoMet, the methyl group donor for transmethylation reactions (0.01–1 mM), to reaction mixtures had no significant effect on transcription by polymerase II or III (Fig. 2, lanes 1–5). This suggests that the whole-cell extract probably contains either a saturating concentration of AdoMet or sufficient precursors and enzymes to synthesize all the AdoMet required for optimum transcription under the conditions used here, even though the extract is concentrated by ammonium sulfate precipitation and subsequently dialyzed.

To demonstrate that the AdoHcy effect on polymerase II transcription is not unique to the Ad2 major late promoter, a recombinant containing the human β-globin gene promoter was transcribed in experiments similar to the one described in Fig.

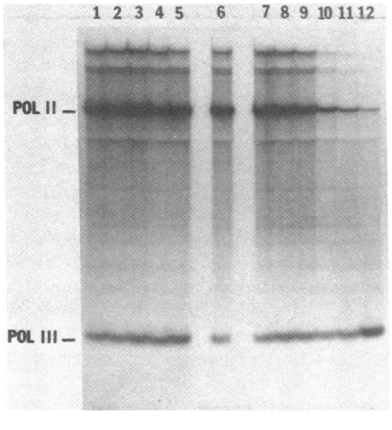

FIG. 2. Effects of AdoMet (lanes 1–5) and AdoHcy (lanes 7–12) on RNA polymerase II (POL II) transcription. *Kpn* I-cleaved pH3B*R template DNA transcribed in a standard reaction mixture gives a 2.5-kb RNA polymerase II run-off transcript and the VA RNAs approximately 160 nucleotides long transcribed by RNA polymerase III (POL III; lane 6). Lanes: 1–5, AdoMet at 10 μM, 100 μM, 250 μM, 500 μM, and 1 mM, respectively; 7–12, AdoHcy at 10 μM, 50 μM, 100 μM, 250 μM, 500 μM, and 1 mM, respectively. Reaction mixtures were incubated for 60 min and, after extraction and glyoxalation, RNAs were resolved in a 1.4% agarose gel.

2. The results were identical to those obtained with the Ad2 late promoter clone (Fig. 3A, lanes 3 and 4).

To determine whether the AdoHcy effect on transcription is a function of the size of the transcript or of the time of incubation of the extract, the following experiments were performed. The Ad2 recombinant pϕ4, containing the major late promoter, was cleaved with *Bam*HI and transcribed in the presence (Fig. 3C, lanes 6–10) or absence (lanes 1–5) of 500 μM AdoHcy for 5, 10, 15, 30, and 60 min. *Bam*HI-cleaved pϕ4 gives rise to a short polymerase II run-off transcript of the expected size, 375 nucleotides, the synthesis of which is sensitive to AdoHcy, even at the earliest incubation times. Synthesis of a very short polymerase II run-off transcript 80 nucleotides long, analyzed by electrophoresis through 12.5% acrylamide/7 M urea gels, is also inhibited by AdoHcy (data not shown).

In another experiment, long polymerase III transcripts were synthesized in the presence or absence of 500 μM AdoHcy by using the recombinant pVAtk10, which contains the VA I promoter inserted in the 5′ flanking region of the herpes thymidine kinase gene. Polymerase III-catalyzed transcripts of 1.6, 1.2, 0.6, and 0.4 kb were detected (Fig. 3B, lanes 1 and 2). These RNAs arise by transcription initiation at the VA I promoter and termination at various sites in the thymidine kinase gene (unpublished results). Transcription of these RNAs is not affected by addition of AdoHcy (compare lanes 1 and 2). However the appearance of the larger transcripts in lanes 1 and 2 is inhibited by AdoHcy. Synthesis of these transcripts, though, is also sensitive to α-amanitin at 0.5 μg/ml, indicating that they are products of RNA polymerase II (unpublished results). Lanes 3 and 4, Fig. 3B, show a 0.9-kb run-off transcript transcribed from a promotor contained in an *Alu* I repeat sequence present in a 9.3-kb *Bam*HI restriction fragment that also contains the human β-globin gene. This RNA can be transcribed *in vitro* by polymerase III (unpublished results), and its synthesis is also insensitive to AdoHcy. These results confirm that the AdoHcy effect is specific for transcription by RNA polymerase II and show that it is not related to the size of the transcript.

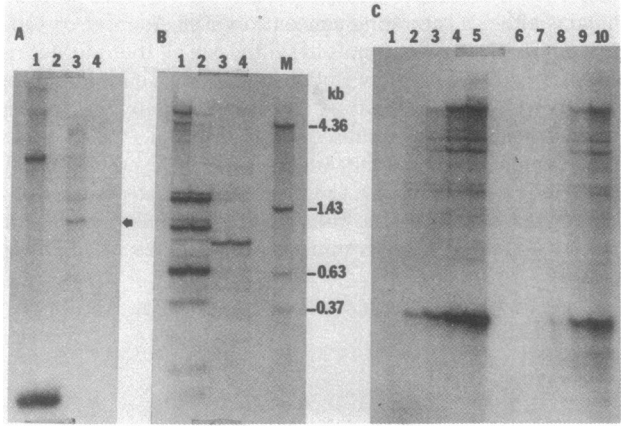

FIG. 3. (A) Effect of AdoHcy on transcription from two mammalian RNA polymerase II promoters. Lanes: 1 and 3, transcription products from *Kpn* I-cleaved pH3B*R and an *Eco*RI digest of a recombinant clone containing a 7.5-kb *Hin*dIII restriction fragment with the human β-globin gene promoter, respectively; 2 and 4, the corresponding transcription products synthesized in the presence of 1 mM AdoHcy. Arrow, position of the 1.4-kb run-off transcript from the β-globin promoter. (Bands at the bottom of lanes 1 and 2 are the VA RNAs transcribed by RNA polymerase III.) Reactions were as described in Fig. 2 except that incubation was for 30 min. (B) Synthesis of long RNA polymerase III transcripts in the presence (lanes 2 and 4) and absence (lanes 1 and 3) of 500 μM AdoHcy. Incubation was for 30 min. Lanes: 1 and 2, transcription products from *Hin*dIII-cleaved pVAtk10; 3 and 4, run-off transcript from a *Bgl* II digest of a 9.3-kb *Bam*HI restriction fragment containing the human β-globin gene and flanking *Alu* I repeated sequences; M, marker RNA prepared by using pϕ4 DNA cleaved with various restriction enzymes as templates. (C) Kinetics of accumulation of short RNA polymerase II transcripts in the presence (lanes 6–10) and absence (lanes 1–5) of 500 μM AdoHcy. *Bam*HI-cleaved pϕ4 DNA was used as template in standard reaction mixtures incubated for 5 (lanes 1 and 6), 10 (lanes 2 and 7), 15 (lanes 3 and 8), 30 (lanes 4 and 9), or 60 (lanes 5 and 10) min.

AdoHcy Does Not Prevent Elongation of Nascent RNAs. The following experiment was carried out to determine whether AdoHcy prevents elongation by RNA polymerase II once initiation has occurred. RNA transcribed from *Kpn* I-cleaved pH3B*R was pulse labeled with 10 μM GTP/[α-^{32}P]GTP for 10 min in a standard reaction mixture without AdoHcy. During this time, nascent RNA chains elongated to various extents were labeled. After the pulse, the reaction was chased with 1 mM Mg^{2+}/GTP in the presence or absence of 500 μM AdoHcy for 0, 5, 10, or 15 min (Fig. 4). If AdoHcy inhibits elongation by polymerase II, the accumulation of labeled 2.5-kb run-off transcripts observed in the presence of AdoHcy (Fig. 4, lanes 1, 3, 5, and 7) should differ from that obtained in the absence of AdoHcy (lanes 2, 4, 6, and 8). However, the intensity of the band resulting from the 2.5-kb run-off transcript is increased by an identical amount (threefold after 15 min) in both cases and with identical kinetics. As a control, parallel incubations in the presence or absence of 500 μM AdoHcy were pulse labeled without chasing for 15 or 30 min (lanes 9–12). These results show that AdoHcy must be present during initiation of transcription by polymerase II to prevent accumulation of the 2.5-kb transcript and that elongation is not affected.

It should be noted that transcripts apparently initiated at sites other than the major late promotor (these include T-A-T-A-A-like sequences in pBR322, as well as the ends of the DNA) are also sensitive to AdoHcy (see Fig. 3C, lanes 1–5 vs. lanes 6–10). This result suggests that initiations at many sites by the RNA polymerase II contained in the whole-cell lysate are sensitive to AdoHcy.

A trivial explanation for the AdoHcy effect on polymerase II transcription might be suggested if AdoHcy inhibits transcription by the purified enzyme. Therefore, the effect of AdoHcy on transcription by purified HeLa RNA polymerase II (a gift of A. Fire and M. Samuels) was tested in an *in vitro* assay using denatured calf thymus DNA as template (27). Although α-

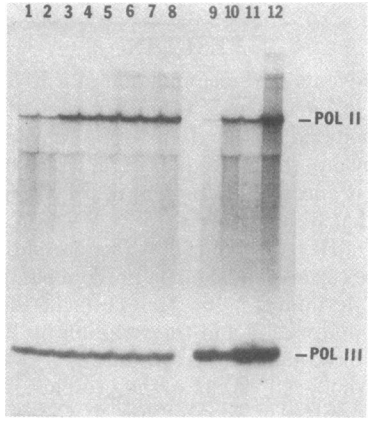

FIG. 4. Elongation of RNA polymerase II (POL II) transcripts in the presence of AdoHcy. RNA transcribed from *Kpn* I-cleaved pH3B*R was pulse labeled for 10 min with 10 μM GTP containing 10 μCi of [α-^{32}P]GTP and then chased by addition of 1 mM Mg^{2+} GTP with (lanes 1, 3, 5, and 7) or without (lanes 2, 4, 6, and 8) 500 μM AdoHcy for 0 (lanes 1 and 2), 5 (lanes 3 and 4), 10 (lanes 5 and 6), or 15 (lanes 7 and 8) min. Lanes 9–12: parallel incubation mixtures with (lanes 9 and 11) and without (lanes 10 and 12) 500 μM AdoHcy were pulse labeled for 15 (lanes 9 and 10) or 30 (lanes 11 and 12) min. POL III, polymerase I.

amanitin at 1 μg/ml inhibited incorporation of [α-^{32}P]GTP into acid-insoluble material by more than 90%, addition of AdoHcy or AdoMet to 1 mM had no detectable effect (data not shown). These experiments suggest that the component(s) of the polymerase II transcription complex in the HeLa whole-cell extract responsible for conferring AdoHcy sensitivity is not present in purified HeLa RNA polymerase II.

Uncapped RNAs Are Stable in the Whole-Cell Extract. The possibility was considered that prevention of methylation of the 5′-cap structure rendered the RNAs unstable in the HeLa whole-cell extract, accounting for the observed effect by AdoHcy. Initial pulse–chase experiments carried out in the presence of AdoHcy during both pulse and chase indicated that this explanation could be the case only if the degradation of unmethylated transcripts was extremely rapid. In these experiments, no degradation of RNA was observed when transcription was in the presence of 500 μM AdoHcy for 10 min followed by a chase for 0, 2, 5, 10, 15, or 30 min with α-amanitin at 1 μg/ml (data not shown). To address this question more directly, uncapped RNAs were prepared, mixed with capped RNA, and incubated in the extract for various lengths of time (Fig. 5).

Nuclease S1 digestion of run-off RNA transcribed from *Kpn* I-cleaved pH3B*R (Fig. 5*A*, lane 3) and hybridized to the *Hin*dIII fragment B of Ad2 DNA (17.0–31.7 m.u.) removed the unprotected 5′-terminal 190 nucleotides and generated a new 5′-monophosphate terminus (lane 1). Similarly, digestion of the same RNA·DNA hybrid with T1, T2, and pancreatic RNases resulted in a new 5′-hydroxyl terminus 190 nucleotides downstream from the original cap site (lane 2). Lane 4 in Fig. 5*A* shows the transcripts synthesized by *E. coli* RNA polymerase using *Bam*HI-cleaved SV40 DNA as template. This RNA contained a heterogeneous mixture of 5′-triphosphate ends initiated at various sites on the SV40 genome (22). The 3.0-kb band in Fig. 5 is capped run-off RNA transcribed from *Bgl* II-cleaved pH3B*R and added to the uncapped RNAs prior to incubation in the extract.

Incubation of these RNAs in the extract for 0, 30, and 60 min revealed no difference in the stability of capped and uncapped RNAs, with the exception of the VA transcripts (Fig. 5*B*, lanes 1–9). The relative instability of the VA transcripts is surprising because the SV40 cRNA population also contains 5′-triphosphate termini and these are stable during the same incubation times. It should be noted that endogenous 28S and 18S rRNAs present in the whole-cell extract, and visualized by ethidium staining of RNAs fractionated through agarose gels, are completely stable for at least 60 min (data not shown). The relatively rapid degradation of the VA RNA thus appears to result from an activity that preferentially degrades a specific class of RNAs.

These results demonstrate that uncapped RNAs with mono- and triphosphate, as well as hydroxyl, groups at the 5′ termini are stable in the whole-cell extract under the conditions described here. Identical results were obtained when capped and uncapped RNAs were incubated in the extract in the presence of AdoHcy (data not shown). In contrast, the 5′-cap structure may be required for stability of mRNA transcripts under certain conditions (4). Thus, the activities responsible for degrading uncapped polymerase II transcripts are either not present or are inactive in the HeLa whole-cell extract as prepared here.

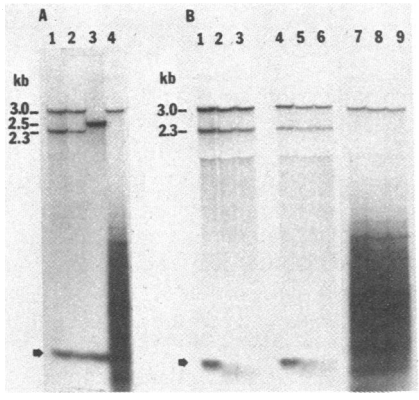

FIG. 5. Stability of capped and uncapped RNAs in HeLa whole-cell extract. (*A*) Capped and uncapped substrates for stability assay. Lanes: 1, 2.3-kb uncapped RNA prepared by hybridization of run-off RNA transcribed from *Kpn* I-cleaved pH3B*R to the *Hin*dIII fragment B of Ad2 DNA followed by nuclease S1 digestion; 2, 2.3-kb uncapped RNA prepared as above except that removal of 5′-terminal nucleotides was by digestion with T1, T2, and pancreatic RNases; 3, 2.5-kb run-off RNA transcribed from *Kpn* I-cleaved pH3B*R DNA template; 4, *E. coli* RNA polymerase transcription products with heterogeneous 5′ termini using *Bam*HI-cleaved SV40 DNA as template. The 3.0-kb RNA in lanes 1, 2, and 4 is capped run-off RNA transcribed from *Bgl* I-cleaved pH3B*R DNA template. (*B*) Capped and uncapped RNAs are stable in the HeLa whole-cell extract. RNAs shown in *A* were incubated in standard reaction mixtures for 0, 30, or 60 min. Lanes: 1–3, capped 3.0-kb RNA and uncapped 2.3-kb RNA (with 5′-monophosphate termini generated by digestion with nuclease S1); 4–6, capped 3.0-kb RNA and uncapped 2.3-kb RNA (with 5′-hydroxyl termini generated by RNase digestion); 7–9, capped 3.0-kb RNA and uncapped SV40 complementary RNA with heterogeneous 5′-triphosphate termini. (Arrows, position of VA RNAs.)

DISCUSSION

The use of a soluble *in vitro* transcription system has provided insight into the mechanism of transcription initiation of mRNAs in eukaryotic cells. The hypothesis that methylation of the cap structure is coupled to initiation of transcription by RNA polymerase II is supported by several observations. First, AdoHcy inhibits transcription initiation by polymerase II but has no effect on transcription by polymerase III. Second, AdoHcy does not inhibit elongation of nascent RNA chains that are already capped. Finally, the AdoHcy effect is very unlikely to result from increased instability of unmethylated cap structures; a cap structure is not required at all for a stable RNA in this system, and no degradation of the RNA synthesized in the presence of AdoHcy could be detected.

It should be emphasized that the experiments reported here have been carried out with crude HeLa whole-cell extracts and thus the possibility must be considered that the AdoHcy effect on RNA polymerase II transcription may actually be mediated indirectly by a metabolite of AdoHcy. The inhibitory effect of AdoHcy on polymerase II in the extract is observed only at concentrations greater than 100 μM, while purified HeLa cap methyltransferases are sensitive to AdoHcy at 1–10 μM (6, 9). It is possible, however, that most of the exogenous AdoHcy is rapidly metabolized in the crude extract. AdoHcy can be hydrolyzed to adenosine and L-homocysteine by the cellular enzyme S-adenosylhomocysteine hydrolase (EC 3.3.1.1) in a reversible reaction (28). Incubation of the extract with 1 mM adenosine does, in fact, result in inhibition of polymerase II transcription. However, since 500 μM adenosine does not significantly inhibit transcription, in contrast to 500 μM AdoHcy, which inhibits RNA polymerase II initiation by a factor of 0.25 to 0.33 (Fig. 2, lane 11), the inhibition by AdoHcy does not result from adenosine generated by hydrolysis of AdoHcy (unpublished results). Perhaps related to this *in vitro* effect observed at 1 mM adenosine are *in vivo* experiments indicating that adenosine toxicity in cultured human cell lines (29) results from elevated levels of AdoHcy generated by reversal of the AdoHcy hydrolase reaction. At present, however, we cannot demonstrate a direct relationship between the adenosine and AdoHcy effects in the crude extract. Alternatively, we cannot rule out the possibility that AdoHcy affects protein(s) not involved in cap formation but whose function(s) is required during

initiation by RNA polymerase II.

The suggestion that cap formation is coupled to initiation by RNA polymerase II is consistent with a mechanism that ensures that all mRNA precursors are properly modified at the 5′ end at an early stage of transcription. That cap formation occurs very early in the synthesis of a nascent RNA chain is supported by other observations. Previous *in vitro* transcription experiments using nuclei isolated from Ad2-infected HeLa cells suggested that cap formation and initiation of transcription by RNA polymerase II are linked (10). Analysis of the 5′-terminal capped undecanucleotide of RNA synthesized *in vitro* from the Ad2 major late promotor showed that the amount of radioactivity in m^7GMP is equivalent to the amount of radioactivity in any other single nucleotide. Post-transcriptional capping of RNA transcripts initiated *in vivo* and elongated *in vitro* would have resulted in a greater-than-equimolar amount of label in m^7GMP. Consistent with these *in vitro* results are *in vivo* experiments suggesting early and complete capping of nascent RNA polymerase II nuclear transcripts (30).

Cap formation has been extensively studied in animal viruses with virion-associated RNA polymerases (1). In the vesicular stomatitis virus system, *in vitro* experiments have indicated that capping and methylation are tightly coupled to mRNA synthesis (31). In the cytoplasmic polyhedrosis virus system, capping appears to be a pretranscriptional event, although methylation of the caps is not required for mRNA synthesis (14). In addition, AdoMet and AdoHcy stimulate transcription in this system. It has been proposed that the presence of AdoMet or similar molecules at the active site of the methyltransferase may induce a conformational change in the enzyme that is communicated to the RNA polymerase in the polyhedrosis transcription complex, thereby allowing initiation of mRNA synthesis (14). In contrast, in the reovirus system, capping enzymes appear to be independent of the RNA polymerase (32), although under certain conditions cap formation occurs at an early stage of transcription initiation (33).

Taken together, the results described above suggest a close association between the 5′-modification enzymes and RNA polymerase II during initiation of transcription. One model would require an obligate linkage between 5′-cap methylation and transcription initiation. However, the finding that full-length run-off transcripts from the Ad2 major late promotor synthesized in the presence of 500 μM AdoHcy are greater than 90% 5′ terminated by GpppA strongly suggests that cap methylation is not essential for transcript formation (W. Filipowicz, H. Ernst, and A. Shatkin, personal communication). An alternative model, consistent with all the available data, postulates that interaction of AdoHcy with a component of the RNA polymerase II initiation complex, presumably a methyltransferase, results in a conformational change that can prevent initiation, perhaps by causing dissociation of the complex. Our *in vitro* results, then, may simply reflect a perturbation of the normal situation. For example, AdoHcy, generated by cleavage of AdoMet during the methyl transfer reactions, may bring about dissociation of these enzymes from the transcribing polymerase, perhaps during the early stages of elongation. Thus, the presence of high levels of AdoHcy prior to initiation may cause premature dissociation, thereby blocking initiation. Note that this model does not actually require 5′-terminal methylation as a prerequisite for initiation. In addition, although optimum transcription rates may require a complete initiation complex, this model does not preclude transcription at a reduced efficiency in the absence of proper interaction of all the components of this complex.

The postulated interaction between cap formation enzymes and RNA polymerase II as components of the initiation complex suggests a possible mechanism for regulating the overall rates of mRNA transcription in cells, perhaps by regulating the concentrations of AdoMet or AdoHcy. A recent report on poliovirus-induced inhibition of transcription by RNA polymerase II in whole-cell extracts prepared from infected cells (34) may be related to such a mechanism. Poliovirus is a rare exception among eukaryotic viruses in that its mRNAs do not contain a 5′-cap structure (35). It is interesting to speculate that this inhibition is mediated by interfering with cap formation in the infected host cell.

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