Dinucleotide Priming of Transcription Mediated by RNA Polymerase II*

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Mammalian RNA polymerase II was shown to utilize dinucleoside monophosphates for priming of promoter specific RNAs. In a reconstituted system containing purified polymerase and HeLa cell fractions, dinucleotides were incorporated by complementarity with template sequences at sites of the adenovirus major late and adenovirus early region IV promoters. Incorporation was shown by label transfer experiments and by determining the size of 5'-terminal RNAse T1-resistant oligonucleotides. All 16 dinucleotides were tested for priming of RNA chains at the major late promoter. RNA polymerase II initiated with various primers over a contiguous region of 9 bases, centered around the in vivo initiation site. We suggest that the polymerase drifts or oscillates over this region. Using a dinucleotide challenge protocol, the rate of initiation at the major late promoter was measured following preincubation of the template DNA with RNA polymerase II and factors. Initiation with ATP was 90% complete within the 1st min after addition of nucleotide triphosphates. Stimulation of transcription by dinucleotides was not observed, due to this rapid initiation. The 5'-hydroxyl terminus of dinucleotide-primed RNAs remained unmodified. Although transcripts initiated with ATP were rapidly capped in whole cell extracts, ATP-primed RNA synthesized in the reconstituted system retained free 5'-terminal phosphates. Thus, capping was not essential for synthesis of long runoff RNAs.

In recent years, transcription mediated by eukaryotic RNA polymerase II has been faithfully reproduced in vitro (1, 2). The primary criterion for this reaction has been the formation of a correct 5' end, as determined by comparison with the sequence of in vivo RNA. Analysis of mutants has shown that many of the same sequences promote initiation of transcription both in vivo and in vitro (3–8). However, little is known of the details of the reaction. Several factors are required in addition to purified polymerase II to obtain accurate transcription (9–12), suggesting that the complete reaction pathway is complex.

We have begun an analysis of the transcription reaction, using a reconstituted system consisting of purified RNA polymerase II and partially purified HeLa cell protein fractions (12). The transcriptional activities in these fractions each titrate linearly with protein concentration and yield single peaks of activity after sedimentation through sucrose gradients. Employing a preincubation-pulse-chase protocol, several steps in the transcription reaction have been resolved (13). Components of the transcriptional apparatus can associate with the DNA to form template-specific "activated" complexes during preincubation in the absence of nucleotides. During the pulse, these complexes rapidly incorporate radioactive nucleotides into RNA chains. In addition, the activated complexes are resistant to a challenge by inhibitory concentrations of DNA. Stable protein-DNA complexes have also been observed in template competition experiments with eukaryotic RNA polymerases II and III (14–16).

A variety of physical and kinetic studies of Escherichia coli RNA polymerase has revealed a multistep pathway leading from free enzyme to initiation (17). Several intermediates occur following binding of RNA polymerase to template DNA (18, 19); the final "open" complex appears to involve invasion of the double helix by the enzyme (20–22). The mechanism of initiation by E. coli RNA polymerase has been studied by the use of special primers in place of nucleoside triphosphates. The bacterial RNA polymerase can utilize a variety of short oligonucleotides to start RNA transcripts (23, 24). In particular, dinucleotides specifically stimulate initiation at promoter sites having complementary sequences; different dinucleotides can be used to alter the 5' termini of transcripts from a given promoter (25–28).

Eukaryotic RNA polymerases I and III have recently been shown to use dinucleotides for RNA chain initiation (29, 30). As yet, incorporation of dinucleotides by RNA polymerase II at promoter sites has not been reported. However, the apparent stuttering of this enzyme during transcription of the polyoma early region suggests that RNA polymerase II can carry out transcription with oligonucleotide primers (31).

Transcription by RNA polymerase II differs from transcription by the other eukaryotic RNA polymerases in that the RNA polymerase II transcripts are modified by the addition of a cap (32). A majority of the promoter-specific RNAs synthesized in soluble cell extracts are also capped (1, 2). RNAs synthesized in isolated cytoplasmic polyhedrosis virus are similarly capped; in this system, transcription and capping appear to be mechanistically coupled (33). The rapid kinetics of capping heterogeneous nuclear RNAs in vivo (34) together with the observation that RNA polymerase II transcription is inhibited in vitro by S-adenosylhomocysteine (35).

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1. J. K. Wilkinson, K. G. Miller, and B. Sollner-Webb, personal communication.
have led to the speculation that cap formation is obligatory for transcription. We describe here the use of dinucleoside monophosphates to probe transcription initiation by RNA polymerase II.

**MATERIALS AND METHODS**

Unlabeled and 32P-labeled high performance liquid chromatography-purified nucleoside triphosphates were purchased from ICN and New England Nuclear, respectively. Dinucleotides were from Sigma, ICN, or Boehringer Mannheim. The identities of all dinucleotides were confirmed by thin layer chromatography on polyethyleneimine-cellulose, both in 0.1 N acetic acid and in 0.5 M NaCl (36). RNAses T1, T2, and T4 were purchased from Calbiochem, RNase A was from Sigma, and proteinase K was from Boehringer Mannheim. Nitrocellulose filters, with 25-mm diameter and 0.45-μm porosity, were from Schleicher and Schuell. E. coli strains infected with phage M13 mp10 or M13 mp11 were a kind gift of J. Vieira and J. Messing (Dept. of Biochemistry, University of Minnesota). End-labeled marker RNA was a gift of Harold Drabkin.

**Transcriptional Proteins—**Glycerol gradient-purified calf thymus RNA polymerase II (37), HeLa whole cell extracts (2) and chromatographic fractions [AB], [CB], and [DB] were prepared as previously described (38). Decapped and decapped-heparinized transcription had a specific activity of 2 × 106 units/mg (1 unit = 1 pmol of UMP incorporated in 20 min at 37 °C with denatured calf thymus DNA template). Kedeshe and Chamberlin have shown that RNA polymerase II purified by a similar method and having a comparable specific activity contains from 15–25% active molecules (39). The decay of transcriptional activity during 20 min at 37 °C was 50%.

**DNA Templates—**Plasmid pFLBH contains adenovirus type 2 DNA from 97.1 (HindIII) to 100 map units (EcoRI linker) around the MLP promoter, inserted at positions +321 to +1297 of the major late promoter was inserted between the SalI and HindIII sites of both M13 mp10 and mp11. Phage M13 XH11 (the mp11 recombinant) contains the strand complementary to major late promoter RNA, while M13 XH10 (the mp10 recombinant) contains the opposite strand. For hybridization in vitro synthesized RNA recombinant phase DNA was purified by CsCl gradient ultracentrifugation of pro tease-digested phase particles. This density gradient step was important for reproducible hybridization by different phase preparations.

The EcoRI-XmaI fragment of pHinI5 (3231 to +250 of the EIV promoter) was inserted between the EcoRI and XmaI (SmaI) sites of both M13 mp10 and mp11. The resulting recombinants (M13 XE10 and M13 XE11) were prepared as phase DNAs. M13 XE11 hybridizes to EIV RNA, while M13 XE10 contains the opposite DNA strand.

**In Vitro Transcription—**The three-stage protocol and reaction conditions are described in detail in the preceding paper (13). Briefly, RNA polymerase II (26 units), fractions [AB] (1 μl), [CB] (5 μl), [CD] (3 μl), and [DB] (2 μl) were preincubated for 1 h with template DNA (at 10 μg/ml) in 20 μl, 5 μl of pulse mix were then added, giving final concentrations of 30 μM ATP, CTP, and UTP and 1 μM [32P]-GTP (3000 Ci/mmol). In some cases, the pulse phase also contained one of the 16 dinucleotides, at 2 mM final concentration. After a short pulse of 3 or 4 min, 5 μl of chase nucleotides were added to give final concentrations of 1 mM for each of the four NTPs. Concentrations of glycerol and salts were maintained throughout each phase of the reaction. The phases were in all cases adequate for complete elongation of runoff transcripts.

**Analysis of RNA—**Reactions were stopped and the nucleic acids purified as described previously (40). Two rounds of ethanol precipitation were required to remove the large amount of unlabeled nucleic acids added in the first round.

Short runoff were analyzed on 0.2 mm thick, 8% polyacrylamide-urea sequencing-type gels. Long runoffs were resuspended in 100 μl of 50 mM Hepes-NaOH, pH 7.0, 1 mM EDTA for M13 analysis. M13 selection was performed according to Hansen and Sharp (41). To each reaction, 0.2 μg of recombinant M13 phage DNA was added, and NaCl was added to 0.75 M. Hybridization mixtures were heated to 70–80 °C for 5 min, cooled to room temperature and incubated at 30 °C. Hybridizations were stopped with 200 μl of cold quench buffer (10 mM Hepes-NaOH, pH 7.5, 1 mM EDTA, 0.2 M NaCl). Hybrids were treated with 5 units of RNase T1, a single-strand RNA-specific endonuclease, for 30 min at 30 °C, the T1 was subsequently destroyed by digestion with 50 μg of proteinase K for 90 min at 30 °C.

The hybrids were filtered slowly (about 1 ml/min) through nitrocellulose filters. Filters were washed with 5–10 ml of quench buffer. The bound nucleic acids were eluted by boiling for 5 min in 1.5 ml of 2 mM EDTA, pH 7.0, plus 25 μg of carrier tRNA, and cooled rapidly in ice water. The nucleic acids were precipitated twice from ethanol, and resuspended in 80% formamide, 0.05 M Tris-borate, pH 8.3, 1.25 mM EDTA for direct acrylamide gel electrophoresis.

For preparation of T1 oligonucleotides, the M13-selected RNA dissolved in 2 mM EDTA, pH 7.0, was heated for 5 min at 90 °C, quick chilled in ice water, and digested with 5–10 units of RNase T1 for 2 h at 30 °C. 50 μl of water freshly saturated with diethyl pyrocarbonate were added to inactivate the RNase, and reactions were lyophilized three times with 50–50 water washes. The resulting oligonucleotides were resuspended in 80% formamide, 0.05 M Tris-borate, pH 8.3, 1.25 mM EDTA and electrophoresed on 0.2-mm thick, 15% polyacrylamide-urea gels.

For RNase U2 digestion, RNAs dissolved in 2 mM EDTA, pH 7.0, were digested with RNase T1 as above. After the T1 incubation, sodium citrate, pH 3.5, was added to 20 mM. After another round of heating and rapid cooling, 0.02 unit of RNase U2 was added for 15 min at 30 °C. The digestion products were lyophilized, dissolved, and electrophoresed as described above.

Decapping was performed by periodate oxidation and β-elimination with lysine (as described; see Ref. 41) before M13 selection. Phosphatase treatments were performed after M13 selection, with 16 units of bacterial alkaline phosphatase (Worthington) for 45 min at 65 °C in Tris, pH 7.9, 1 mM EDTA, 0.05% sodium dodecyl sulfate. For unknown reasons, late promoter RNA was highly resistant to phosphatase at 30 °C.

For label transfer analysis, oligonucleotides from complete T1 digestion of preincubation-pulse-chase RNAs were gel purified, and digested either with RNase T2 (1 unit, 37 °C overnight in 20 mM ammonium acetate, pH 4.5) or with RNase A (0.4 μg, 37 °C for 60 min in 10 mM Tris, pH 7.4, 1 mM EDTA). The digestion products were resolved by chromatography on cellulose thin layers in one or two dimensions (42).

To confirm that the decapping reaction had worked, [α-32P]TP-labeled late promoter RNA was synthesized by a whole cell extract in a simple 3-h reaction. The 5′-terminal 41-nucleotide fragment was isolated as described (43). Aliquots were removed for decapping and/ or phosphatase treatment. The products of complete RNase T2 digestion were resolved by two-dimensional thin layer chromatography. From untreated, decapped, and decap + phosphatase reaction spots were observed with the mobilities expected for GpppAmCp, pppAmCp, and AmCp, respectively (2′-O-methylation protected the A from T2 cleavage). A spot possibly consisting of m3AmCp was also observed.

Quantitation— Autoradiograms obtained with response-linearized (preflashed) film were quantitated by densitometry and planimetry (12). For estimates of absolute transcriptional efficiency, T1 oligonucleotide bands were excised from acrylamide gels and counted for Cerenkov radiation. The number of RNA chains initiated in the pulse was calculated from the specific activity of the labeled nucleotide.

**RESULTS**

Transcription reactions were performed using a reconstituted system consisting of purified RNA polymerase II supplemented with HeLa cell fractions [AB], [CB], and [DB] (Ref. 12, and see Fig. 1A). The three-stage protocol described by Fire et al. (13) was used to resolve the reaction into several kinetic steps. As shown in Fig. 1B, RNA polymerase II, factors, and template DNA were first preincubated, allowing

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4 U. Hansen, and P. A. Sharp, manuscript in preparation.
accurately reproducing the heterogeneity observed in vivo.

Formation of "activated" complexes. Radioactive nucleotides were added for a brief pulse, during which elongation proceeded only a short distance. An ensuing chase with excess unlabeled nucleotides allowed the completion of long runoff RNAs.

To examine initiation by the activated complexes, pulse nucleotide concentrations were varied. The template for these experiments contained the Ad5 early region IV promoter (see Fig. 2B). Previous work with this promoter has shown that the site of initiation in vivo exhibits heterogeneity over a seven-nucleotide region in the whole cell extract, thereby accurately reproducing the heterogeneity observed in vivo (44). Moreover, the relative amounts of the various termini depend on the nucleotide concentrations in the reaction. Thus, high UTP concentrations favor the use of U initiation sites (residues -6 to -1), while high ATP concentrations favor initiations at A (residue +1).

Analogous results were obtained with the reconstituted system using the three-stage protocol. EIV runoff RNAs were synthesized under conditions of either high ATP or high UTP concentration in the pulse. These RNAs were analyzed for differences at the 5' end by the method of Hansen and Sharp.

For this analysis, the long runoff RNAs were hybridized to a single-stranded promoter-proximal RNA, which was selected from a HindIII-cleaved, end-labeled SV40 DNA. The 200 nucleotide marker was a truncated runoff RNA from the major late promoter.

RNase T1, Filter, Etute

Electrophoresis

250 nucleotides.
subjected to this shift, these transcripts must be initiated during the pulse phase.

Initiation with Dinucleotides at the EIV Promoter—At low nucleotide concentrations, E. coli RNA polymerase will utilize dinucleotides to initiate RNA chains, incorporating the dinucleotides according to base-pairing rules (24–28). To test whether RNA polymerase II behaved similarly, 5’-shift experiments were performed using dinucleotides with the EIV promoter. When UpU was added to a transcription pulse, the resulting RNA migrated more slowly than the control RNA initiated at low nucleotide concentrations (Fig. 2C, lanes 1 and 7). This UpU RNA co-migrated with RNAs initiated at high [UTP] (compare lanes 3 and 7), indicating that initiation in the presence of the dinucleotide had occurred in the stretch of T residues upstream of +1. A similar upstream shift was observed with CpU (Fig. 2C, lane 6), as expected from the sequence of the EIV promoter. This shift in mobility was specific to UpU and CpU, as neither ApC nor CpA visibly affected the migration of the EIV RNA (Fig. 2C, lanes 4 and 5). A shift of one nucleotide (expected if CpA priming occurred) would probably not be detected with this gel system. However, the fact that addition of either ApC or CpA stimulated labeling of the EIV RNA in a short pulse indicates that these dinucleotides probably did prime transcription at the EIV promoter.

Initiation with Dinucleotides at the Major Late Promoter—The MLP is more efficient in a whole cell extract than is the EIV promoter (44). In further contrast to EIV, initiation at the MLP occurs in vivo and in vitro at a single A residue (nucleotide 6039 of adenovirus type 2). Analysis of initiation at the MLP is facilitated by the presence of a large RNase T1-resistant oligonucleotide containing the 5’-terminus (45, 46).

This oligonucleotide was analyzed directly using the following procedure. The MLP was transcribed using the threestage protocol described above. The short pulse with low concentrations of the radioactive nucleotide allowed incorporation of label only near the 5’-terminus. After completion of chains during the chase, RNA was hybridized to a single-stranded M13 recombinant spanning the MLP (M13 XH11, containing sequences from −261 to +197 of the MLP). Remaining single-stranded RNA was digested with ribonuclease and the RNA/DNA hybrid was selected on nitrocellulose filters. RNA was released from the hybrid and digested to completion with RNase T1. The labeled oligonucleotide products yielded a simple pattern when resolved by electrophoresis in a 15% acrylamide, 8 M urea gel (Fig. 3A, lane 1). Five major bands, designated A–E, were observed. Bands A, B, C, and D, migrated in the size range of 1 to 6 bases relative to a size marker, while band E migrated in the range of 9–13 bases. Tentative assignment of these oligonucleotides is shown in Fig. 3B. Since the label was [α-32P]GTP and each oligomer contained only one G residue (at the 3’ end), the intensity of each band was proportional to the amount of the corresponding oligomer synthesized during the pulse. The gradient of band intensities ran E-D-B-(A,C). This agreed perfectly with the assignments based on size. Long exposure revealed a faint T1 product running at 16 nucleotides. This longer oligomer corresponded to the 16-mer ending at position +62. Its low intensity suggests that very few polymerase molecules reached that position during the pulse.

In contrast to the results obtained with the EIV promoter, varying nuclease triphosphate concentrations had no effect on the site of initiation of the MLP. Concentrations of CTP or UTP as high as 1 mM did not result in initiation at C and T residues adjacent to +1 (data not shown).

Examination of the MLP sequence (Fig. 3B) suggested the dinucleotides ApC and CpA as good candidates for priming RNA synthesis. Addition of either dinucleotide to the pulse resulted in a shift of the putative 5’-terminal oligonucleotide (Fig. 3A, lanes 2 and 3). The internal oligomers (A–D) migrated identically in each reaction. As predicted from the sequence, the CpA band (E12) migrated more slowly than the ApC band (E11). The ApC band, in turn, migrated more slowly than the control band E, which presumably resulted from initiation with ATP at position +1. A possible reason for the faster migration of band E was the presence of phosphates at the 5’ end of this oligomer. The effect of phosphatase treatment on the mobility of these oligonucleotides was therefore investigated.

RNAs primed without dinucleotides (control), or with either ApC or CpA, were treated with phosphatase before complete RNase T1 digestion. In this manner, only 5’-terminal phosphates should be susceptible to hydrolysis. As expected, phosphatase digestion did not affect the migration of internal T1 oligomers (Fig. 3C, bands A–D). The ApC band (E11) and CpA band (E12) were likewise not affected (lanes 6 and 10), whereas band E was affected by phosphatase (lane 14); after treatment, it co-migrated with band E12. Thus, 5’-terminal phosphates were present on ATP-primed but not on ApC- and CpA-primed RNA.

The phosphatase sensitivity demonstrated that the control RNA was not capped. In contrast, previous results have shown that RNA synthesized in the whole cell extract is efficiently capped (2). The latter results were confirmed using RNA prepared in a whole cell extract by the three-stage protocol. This RNA was analyzed by M13 selection followed by complete RNase T1 digestion. Internal oligomer bands A–D were identical with those of the reconstituted system (Fig. 3C, lanes 1 and 13). However, the largest whole cell extract T1 oligomer (E) migrated more slowly than the corresponding band E from the reconstituted system. The mobility of E was not affected by phosphatase treatment (lane 2). Chemical decapping of the whole cell extract RNA prior to T1 cleavage altered the mobility of band E, such that it co-migrated with bands E (lane 3). Decapped E was also rendered phosphatase sensitive; the combined decap + phosphatase treatment altered the mobility of band E, such that it co-migrated with ApC band E11 (compare lanes 4 and 5). Decapping had no effect on RNAs made in the reconstituted system (lanes 7, 11, and 16).

The following structures, with initiating nucleotides underlined, were postulated for the 5’-terminal oligonucleotides.

In the whole cell extract:

\[
\text{E, (ATP-initiated):} \quad \text{GpppACUCUCUUCCGp}
\]

In the reconstituted system:

\[
\text{E (ATP-initiated):} \quad \text{GpppACUCUCUUCCGp}
\]

\[
\text{E11 (ApC-initiated):} \quad \text{ACUCUCUUCCGp}
\]

\[
\text{E12 (CpA-initiated):} \quad \text{CAUCUCUUCCGp}
\]

These T1 oligonucleotides were further examined by digestion with RNase U2, which cleaves after A and G residues. As expected, RNase U2 digestion converted each of the four oligomers, E, E11, and E12, to a single 10-mer (data not shown). The sensitivity of the capped oligomer (E1) to RNase U2 indicated that none of the capped A was 2’-O-methylated. The extent of methylation at the 7-position of guanine in the cap was not ascertained.

Definitive proof of dinucleotide incorporation was obtained by the following label transfer experiments. RNA was made with control nucleotides, or with added ApC or CpA in the
Fig. 3. Analysis of MLP transcripts initiated with ATP or with dinucleotides. A, T1 oligonucleotide analysis of major late promoter RNA. Long runoff RNA was synthesized using the three-stage protocol with linearized pFLBH as template. The standard pulse nucleotides had the following additions: lane 1, no additions; lane 2, ApC; lane 3, CpA. RNAs were hybridized to single-stranded M13 XH11 DNA, containing coding sequences between -261 and +197 of the MLP. The hybrids were RNase-treated and filter-selected as in Fig. 2A. After elution from the filter, the RNA was digested to completion with RNase T1. The products were resolved on a 15% sequencing-type gel. Bands A–D refer to T1 oligonucleotides whose sequence is in B. Bands E, E11, and E12 correspond to the putative 5′-terminal oligonucleotides resulting from the control, ApC, and CpA reactions, respectively. B, sequence around the adenovirus type 2 major late promoter cap site (m.u., map units). Initiation in vivo occurs exclusively at the indicated A residue (45, 46). Slash marks indicate sites of cleavage of RNA transcripts by RNase T1; A–E designate the 5′-proximal T1 oligonucleotides (see A). C, sensitivity of late promoter RNAs to decapping and phosphatase. Long runoff RNAs synthesized with the three-stage protocol as in A were hybridized to M13 XH11, truncated with RNase T1, and filter purified. Aliquots were removed and treated with decapping and/or phosphatase reagents as described under "Materials and Methods." Products in lanes 1–4 were made in a whole cell extract; those in lanes 5–16 were made in the reconstituted system. The standard pulse nucleotides received either no additions (lanes 1–4 and 13–16), ApC (lanes 5–8), or CpA (lanes 9–12). Samples were treated with phosphatase (lanes 2, 6, 10, and 14), decapped (lanes 3, 7, 11, and 15), or decapped followed by phosphatase treatment (lanes 4, 8, 12, and 16). After these treatments, the RNAs were digested to completion with RNase T2 and electrophoresed. Bands A–D refer to oligonucleotides whose sequence is in B. Bands E, E11, and E12, and E, refer to 5′-terminal oligonucleotides synthesized under the different pulse conditions.

| Table I  |
|---|
| Secondary analysis of 5′-terminal oligonucleotides |
| Late promoter runoff RNAs were synthesized using the three-stage protocol. Each sample was scaled to five standard reaction volumes. The preincubation contained PstI-cleaved pFLBH at 20 μg/ml. Pulses contained 100 μCi of [α-32P]CTP, UTP, or GTP (1 μM) plus the other three unlabeled nucleoside triphosphates at 30 μM. With each radioactive nucleotide, a reaction was done with these standard pulse conditions, or with the standard pulse plus ApC or CpA. From each reaction, the 5′-terminal RNA T1 oligomer was gel-purified following M13 selection, and was further digested with RNase T2 or A. Products of CTP labeling were separated in two dimensions as described under "Materials and Methods." Products of GTP and UTP labeling were separated only in the first dimension. |
| RNA initiator | Secondary enzyme | Expected | Observed |
| [α-32P]CTP-labeled | | | |
| ApC | RNase T2 | Cp, Up, Gp | Cp, Up, Gp |
| CpA | RNase T2 | Ap, C, Up, Gp | Ap, Cp, Up, Gp |
| ATP | RNase T2 | pppAp, Cp, Up, Gp | Ap, Cp, Up, Gp |
| [α-32P]GTP-labeled | | | |
| ApC | RNase A | Cp | Cp |
| CpA | RNase A | Cp | Cp |
| ATP | RNase A | Cp | Cp |
| [α-32P]UTP-labeled | | | |
| ApC | RNase T2 | Cp, Up | Cp, Up |
| CpA | RNase T2 | Cp, Up | Cp, Up |
| ATP | RNase T2 | Cp, Up | Cp, Up |
| aLoss of 5′-terminal phosphates in this sample apparently resulted from a phosphatase contaminant. |

A Region Accessible for Dinucleotide Priming—The extent of sequences available for priming at the MLP was tested using all 16 dinucleotides. In parallel reactions, each with a different dinucleotide added to the pulse, a variety of patterns was generated. All 16 reactions yielded the same pattern of internal T1 oligomers (Fig. 4, bands A–D). The dinucleotides GpA, ApU, ApG, GpU, GpG, CpG, UpG, and UpU (Fig. 4, lanes 9–16) were not utilized as primers; only the ATP-initiated 5′ oligomer (band E) was observed. The remaining eight dinucleotides yielded different patterns for the 5′ oligomer (lanes 1–8). In each case, some of the ATP-initiated RNA (band E) was observed. As previously shown, ApC and CpA gave an 11-mer and a 12-mer, respectively, corresponding to initiation at positions +1 and −1. CpC, CpU, and UpC each yielded novel bands which were interpretable as resulting
from dinucleotide incorporation according to the DNA sequence: bands of length 15 (CpC), 14 (CpU), and 13 (UpC) were observed. Also in accord with the sequence, CpU gave bands at 10 and 8 nucleotides, and UpC gave a 9-mer. These oligomers ranging from 8 to 15 bases long corresponded to dinucleotide incorporation according to the DNA sequence positions of T1 oligonucleotides; those marked (A)- (E) refer to Fig. 3A. Numbers on the right refer to positions within the DNA sequence (ATP) (lanes 2 (ApC), 4 (GpC), 6 (CpC), and 8 (UpC)). Aliquots from each reaction were removed and further digested with RNase T1 (lanes 2 (ApC), 4 (GpC), 6 (CpC), and 8 (UpC)). Aliquots from each reaction were removed and further digested with RNase T1 (lanes 2 (ApC), 4 (GpC), 6 (CpC), and 8 (UpC)). RNase T1 digestion products were resolved on gels, and the label in the T1 resistant RNA (lane M). Below the gel, the sequence around the in vivo site of initiation (the A residue marked +1) is provided.

Several bands could not be explained by strict complementarity between dinucleotide and DNA. These included 11-mers generated with CpC (Fig. 4, lane 1), UpC (lane 3), and ApC (lane 4). Several bands could not be explained by strict complementarity between dinucleotide and DNA. These included 11-mers generated with CpC (Fig. 4, lane 1), UpC (lane 3), and ApC (lane 4). One possible explanation for these bands was that the dinucleotides were incorporated using complementarity limited to the 5’-terminal oligonucleotides. The +1(5’-ATP) line marks the band corresponding to a 5’-terminal 11-mer initiated with ATP and presumably carrying a triphosphate 5’ end. Nucleotide spacings were compared to an alkaline, formamide hydrolysis ladder of 5’ end-labeled RNA (lane 1M). Below the gel, the sequence around the in vivo site of initiation (the A residue marked +1) is provided.

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At 2 mM, CpA competed effectively for initiation when added at the beginning of the pulse (lane 1). However, when CpA was added after 1 min of pulse, 90% of observed chains had pppA at their 5' terminus; thus, most of the observed chains had already initiated with ATP (lane 2). By 3 min of pulse, almost all chains were ATP-initiated (lane 3). In a control reaction, the addition of CpA to the chase had no effect on the 5' oligomer (lane 4). Similar results were obtained when the ATP, CTP, and UTP concentrations were 300 μM in the pulse, 10 times higher than previously (lanes 5–8). In a separate experiment, it was shown that the incorporation of label into ATP-initiated 11-mers reached a plateau after 2–4 min of the pulse (data not shown). This independently confirmed that initiation occurred synchronously during the standard pulse.

The preincubation-pulse-chase protocol thus functions as a one-round assay for activated complexes. Using this assay, the number of templates and RNA polymerase molecules productively involved in transcription was readily determined by quantitating the amount of radioactivity in 5'-terminal RNase T1-resistant oligonucleotides. 0.1% of templates were transcribed, and 0.1% of the RNA polymerase II molecules functioned in the promoter-specific reaction. The low fraction of participating polymerase molecules did not reflect a poor preparation, as the specific activity of the enzyme was close to the maximum reported values. Kadesch and Chamberlin (38) have directly measured the fraction of active molecules in a similar preparation of RNA polymerase II, using a non-specific phage DNA template. From 15–25% of the enzyme molecules were active in this assay. The fraction of templates used in the specific runoff assay accorded with previous estimates for the efficiency of RNA polymerase II systems (1, 2).

These earlier measurements left open the possibility that some templates were transcribed in multiple rounds; the one-round assay does not suffer from this ambiguity.

**DISCUSSION**

We have shown that RNA polymerase II can utilize dinucleoside monophosphates to initiate faithful transcription at the early region IV and major late promoters of adenovirus. This polymerase therefore shares the ability of *E. coli* RNA polymerase and eukaryotic RNA polymerases I and III to incorporate dinucleotides by sequence complementarity at *in vivo* initiation sites.

The major late and EIV promoters seemed ideal for the study of transcription initiation, as these two promoters represent extremes: initiation *in vivo* occurs at a unique position at the major late promoter but is distributed over several adjacent nucleotides of EIV (41, 46). Initiation *in vitro* at the major late promoter was examined in detail by testing the effects of all 16 dinucleotides on the positions of 5' termini of transcripts. Dinucleotides complementary to sequences from −4 (CpC) to +4 (CpU) primed RNA synthesis. In contrast, initiation at sites other than +1 was never observed by varying nucleoside triphosphate concentrations (data not shown). This suggests that initiation by dinucleotides and by nucleoside triphosphates may proceed by different mechanisms.

The homogeneity of initiation at the major late promoter could be due to the occurrence of an A residue at a mechanistically preferred position (see Baker and Ziff (41) for a similar discussion). *In vivo*, RNA polymerase II initiates most frequently with purines, and with ATP preferentially to GTP. This could reflect preferential binding of ATP to the initiation site of the polymerase. The experiments presented here suggest that RNA polymerase II also has a strong preference for site +1 of the major late promoter, independent of the initiating nucleotide. The efficiency of dinucleotide priming was greatest at positions near +1, as shown by the ability of different dinucleotides to compete with ATP for initiation. Moreover, the novel phenomenon of misincorporation, mediated by sequence complementarity between the second residue of a dinucleotide and the template, was observed only at positions +1 and +2. Dinucleotides might be envisioned to prime the elongation reaction, thereby bypassing initiation events. This would obviate ATP preference, and identify positions on the template available to the elongation site of the polymerase.

**A Region Accessible for Dinucleotide Priming**—The major late promoter results show that a contiguous region of at least 8–9 bases is accessible for dinucleotide priming, centered around the *in vivo* cap site (see Fig. 7). This corresponds to a length of about 30 Å of DNA, or almost a full turn of the helix. It is difficult to picture how a static RNA polymerase aligned at a single base could initiate transcripts over this distance. Such a static complex would have to be capable of elongating RNA primers from eight different sites on the enzyme. An alternative and more likely possibility is that the polymerase drifts or oscillates over 8–9 bases around a preferred site of initiation. In this case, it would be possible to align a single site, for instance the elongation site of the enzyme, with any of eight different positions on the template. This plasticity of initiation is apparently distinctive of RNA polymerase II as compared to *E. coli* polymerase and eukaryotic RNA polymerases I and III (26, 29, 30, 47). A requirement for hydrolysis of the β-γ bond of ATP has been suggested for initiation of transcription by RNA polymerase II (48). This cofactor dependence could be related to oscillation of...
which has unwound approximately one turn of the DNA helix

the enzyme. In any case, the polymerase complex must unwind the DNA to allow base pairing with a complementary primer. E. coli RNA polymerase can initiate from an open complex which has unwound approximately one turn of the DNA helix (20-22). Perhaps dinucleotides prime a similar structure formed by RNA polymerase II and factors.

Rate of Initiation by the Activated Complex—In the accompanying paper, we describe the formation of an “activated” complex by preincubation of template DNA, RNA polymerase II, and factors. This complex was defined by its ability to rapidly begin transcription when presented with nucleoside triphosphates. The activated complex might therefore initiate directly, or might undergo one or more transitions to form the structure immediately preceding initiation. The time required for activated complexes to initiate RNA chains with ATP at the major late promoter was measured using a dinucleotide challenge experiment. Ninety per cent of the observed chains initiated within the 1st min of the pulse. It is worth noting that accumulation of activated complexes occurs over 1-h time interval, but that these complexes initiate quite rapidly. The rate-limiting steps in transcription of the major late promoter by the reconstituted system can thus occur in the absence of nucleotides.

Initiation with ATP was sufficiently fast that stimulation of label incorporation by dinucleotides was not observed in a 4-min pulse. However, stimulation of incorporation by dinucleotides was observed at the EIV promoter. Such stimulation of incorporation was also observed when high concentrations of the initiating nucleoside triphosphates were used. This suggests that initiation at EIV is slower than at the major late promoter.

The Role of Capping—In the experiments presented here, a reconstituted transcription system was used. Nucleoside monophosphates also prime transcription by polymerase II in a whole cell extract (data not shown). Late promoter RNAs primed with dinucleotides had no obvious modifications of the 5’-hydroxyl terminus, either in the reconstituted system (Fig. 3C) or in the whole cell extract (data not shown). Thus, the presence of a cap is not required for and does not enhance transcription by RNA polymerase II. Moreover, RNA initiated with ATP in the reconstituted system has unprotected phosphates at the 5’ terminus. We have already noted that the bulk of guanylyltransferase activity (assayed as described in Ref. 49) is recovered in a fraction not included in the reconstituted system (12). It thus seems highly unlikely that the ATP-initiated RNAs in the reconstituted system were ever capped, although transcripts were efficiently capped in the whole cell extract. Recent experiments with a whole cell wash extract have shown that initiation and some elongation precede capping (50). Together these results indicate that capping plays no direct role in the transcription reaction.

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