Vol. 94, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1980 Pages 553-559

A NEW METHOD FOR DETERMINING THE NUMBER OF RNA POLYMERASES ACTIVE IN CHROMATIN TRANSCRIPTION

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Received April 10,1980

SUMMARY

A new method is described for determining the number of RNA chains which are being actively propagated in an in vitro transcription system. This method employs [^{32}P]-α-cordycepin triphosphate which terminates RNA chain propagation upon incorporation of the nucleotide analogue into an RNA transcript. This method is quicker and subject to fewer problems than end group analysis using [^{3}H]nucleoside triphosphates. The auxin-induced increase in soybean RNA polymerase I activity was examined using this method. At 12, 24, and 48 hours after auxin treatment, the increase in chromatin-bound RNA polymerase I activity is predominantly due to a greater rate of RNA chain elongation rather than to an increase in the number of elongating RNA chains.

INTRODUCTION

The number of elongating RNA chains contributing to in vitro transcription is generally determined by end group analysis utilizing [^{3}H]nucleoside triphosphates as the radioactive label, and the [^{3}H] RNA which is synthesized in vitro is then subjected to alkaline hydrolysis (1, 2, 3). The number of elongating RNA chains is determined from the amount of labeled nucleoside recovered, which represents the number of 3'-termini. This method is subject to a number of potential problems such as the possibility of generating artifactual 3'-termini through the action of chromatin-associated nucleases (1, 2), generating nucleosides from nucleotides during alkaline hydrolysis (2, 4, 5, 6), differential binding and extraction of nucleosides and nucleotides to charcoal (2), and initiation of new RNA chains during the assay period (2). In this paper, a new, simple method is described utilizing [^{32}P]-α-cordycepin triphosphate (3'dATP) for determining the number of propagating RNA chains in an in vitro assay system, and this method is compared to end group analysis utilizing [^{3}H]nucleoside triphosphate.

0006-291X/80/100553-07$01.00/0

3'dATP has been shown to inhibit RNA chain elongation by incorporating into the 3'-terminus of the elongating RNA chain, presumably in the place of ATP, and preventing further RNA chain propagation due to the absence of a 3'-hydroxyl group which is required for continued polymerization (7). Substituting 3'dATP in place of ATP in the RNA polymerase assay should allow the majority of elongating RNA chains to be terminated with a single 3'dAMP residue. The number of elongating RNA chains can be quantitated by measuring the amount of ^{32}P incorporated into RNA using $[^{32}P]$-α-3'dATP as the nucleotide analogue.

Etiolated soybean seedlings which have been treated with the auxin herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), possess high levels of RNA polymerase I activity associated with isolated nuclei or chromatin (8, 9, 10). This enhanced RNA polymerase I activity observed 24 hours after auxin treatment appears to result primarily from increased rates of RNA chain elongation as determined using 3'-end group analysis (11). This paper re-examines and expands our previous results on rates of RNA chain propagation in chromatin isolated from untreated and auxin-treated soybean hypocotyl.

MATERIALS AND METHODS

Soybean seeds (Glycine max var. Wayne) were germinated in the dark in moist vermiculite at 30 C. Auxin-treated material was sprayed with 2.5×10^{-3} M 2,4-dichlorophenoxyacetic acid (pH 6.0) at 72 hours after imbibition. Auxin-treated hypocotyls were harvested at 6, 12, 24, and 48 hours after application of 2,4-dichlorophenoxyacetic acid, and untreated hypocotyls of comparable age were harvested at the same time. Chromatin was prepared as described previously (12) with the exception that 250 mM sucrose was omitted from the homogenization buffer and the concentration of Triton X-100 was reduced to 0.5% (v/v). Chromatin was suspended in ice cold 50 mM Tris-HCl (pH 7.9 at 25 C), 10 mM 2-mercaptoethanol, 1 mM $MgCl_2$, and 50% (v/v) glycerol at a concentration of 0.4-0.8 mg chromatin DNA/ml. DNA concentrations were determined by the method of Burton (13) using calf thymus DNA as a standard.

RNA polymerase was assayed using 2-4 µg of chromatin DNA in a 50 µl reaction mixture containing 50 mM Tris-HCl (pH 7.9 at 25 C), 50 mM $(NH_4)_2SO_4$, 10 mM dithiothreitol, 5 mM $MgCl_2$, 10% (v/v) glycerol, 1 µg/ml α-amanitin, 0.1 mM each of ATP, CTP, and GTP, and 0.01 mM [^{3}H]UTP (0.625 μC_i). When 3'dATP incorporation was assayed, 0.01 mM $[^{32}P]$-α-3'dATP (4.53 μC_i) was substituted for ATP, [^{3}H]UTP was eliminated, and the UTP concentration was increased to 0.1 mM. All assays were performed at 21 C unless indicated otherwise. The reactions were terminated by the addition of 3 ml of 5% (w/v) trichloroacetic acid and 50 mM sodium pyrophosphate. The acid precipitable material was collected on Whatman GF/A filters and radioactivity was determined by liquid scintillation spectrometry.

Nearest neighbor analysis was performed on in vitro synthesized RNA labeled with [^{32}P]-α-3'dATP. The assay volume was increased to 250 µl and contained 8-16 µg of chromatin DNA and 17.4 µC$_i$ of [^{32}P]-α-3'dATP. The assay was terminated as described by Coupar et al. (3), and the RNA associated with the chromatin was subjected to alkaline hydrolysis (3). The hydrolysate (a 10 µl aliquote) was spotted onto a 20 X 20 cm Avicel TLC plate (Analtech, Inc., Newark, Delaware) and chromatographed in two dimensions (14). A mixture of 2',3'-AMP, 2',3'-CMP, 2',3'-GMP, and 2',3'-UMP was chromatographed on the same TLC plate to serve as standards. The chromatogram was autioradiographed on NS-2T x-ray film (Kodak). Nucleotides were located on the TLC plate after chromatography by UV light, scraped into scintillation vials, and counted in a liquid scintillation spectrometer.

[^{32}P]-α-3'dATP (44 C$_i$/mmole) was a generous gift of John Lola and Richard Perullo (New England Nuclear Corp., Boston, Mass.). [^{3}H]UTP was purchased from New England Nuclear Corp. 3'dATP was purchased from Miles Laboratories, and all other nucleotides were from Sigma.

RESULTS AND DISCUSSION

Soybean chromatin isolated with an alkaline buffer as described by Lin et al. (12) contains predominantly RNA polymerase I activity with little or no detectable RNA polymerase II and III activities. A stimulation in chromatin-bound RNA polymerase I activity can be detected as early as 4 hours after soybean hypocotyls are treated with the synthetic auxin, 2,4-dichlorophenoxy-acetic acid, and by 24 hours after application of auxin, chromatin-bound RNA polymerase I has increased 5-10-fold above activity in chromatin isolated from untreated hypocotyl (9). This enhanced RNA polymerase I activity could result from increased numbers of propagating RNA chains, from an increase in the rate of RNA chain propagation, or from a combination of both. To determine which of these possible mechanisms is responsible for the enhanced chromatin-bound RNA polymerase I activity in auxin-treated soybean hypocotyl, we analyzed the numbers of elongating RNA chains and the rates of RNA chain propagation in soybean chromatin isolated from untreated and auxin-treated hypocotyl. This analysis employed [^{3}H]UTP to measure the rates of RNA synthesis and the incorporation of nucleotides into internal residues of RNA chains (11) and [^{32}P]-α-3'dATP to measure the number of RNA chains being propagated. Incorporation of 3'dATP, presumably in the place of ATP, blocks further RNA chain propagation due to the absence of a 3'-hydroxyl group which is required for 3'-5' phosphodiester bond formation (7). Under our conditions used to assay 3'dATP incorporation, the incorporation of [^{3}H]UTP into RNA was inhibited 80-

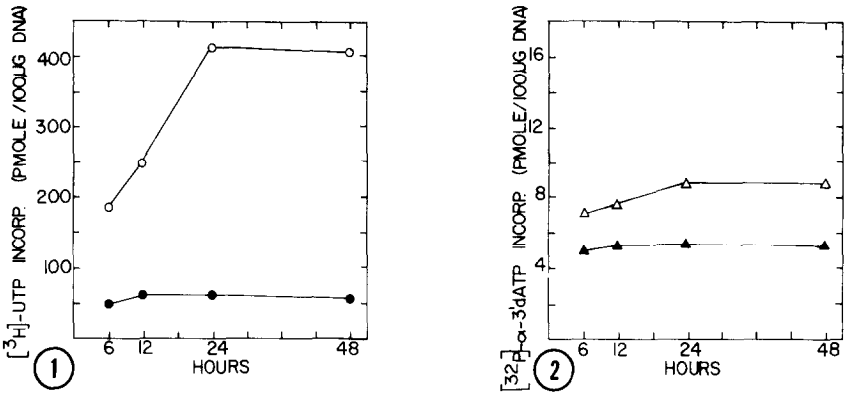

FIGURE 1. Time course for auxin enhancement of chromatin-bound RNA polymerase activity assayed with [^{3}H]UTP. Chromatin was isolated from mature hypocotyls of soybean at the times indicated after auxin treatment or from untreated hypocotyls of comparable age. RNA polymerase activity was assayed using [^{3}H]UTP in the presence of 1 μg/ml α-amanitin as described in Materials and Methods. Assays were conducted for 30 minutes at 21 C. Untreated (●——●); Auxin-treated (○——○).

FIGURE 2. Time course for auxin enhancement of chromatin-bound RNA polymerase activity assayed with [^{32}P]-α-3'dATP. Chromatin was isolated from mature hypocotyls of soybean at the times indicated after auxin treatment or from untreated hypocotyls of comparable age. RNA polymerase activity was assayed using [^{32}P]-α-3'dATP in the presence of 1 μg/ml α-amanitin as described in Materials and Methods. Assays were conducted for 30 minutes at 21 C. Untreated (▲——▲); Auxin-treated (△——△).

90% in the presence of the nucleotide analogue (data not shown). With [^{32}P]-α-3'dATP, a single ^{32}P is incorporated at the 3'-terminus of each RNA molecule which is being propagated *in vitro* since incorporation of this nucleotide analogue prevents further polymerization. Although there is a 5-10-fold increase in the level of [^{3}H]UTP incorporation into RNA at 24-48 hours after auxin application (Figure 1), there is less than a 2-fold increase in the number of RNA chains (^{32}P incorporation) being propagated *in vitro* in chromatin isolated between 6-48 hours after auxin treatment (Figure 2). This result indicates that the increased rate of *in vitro* RNA synthesis occurs primarily from increased rates of RNA chain elongation and supports earlier results obtained with 3'-end group analysis using [^{3}H]nucleoside triphosphates (11). In the present study, the ratio of [^{3}H]UTP incorporation, which represents primarily incorporation of nucleotide into internal portions of RNA chains (11), to [^{32}P]-α-3'dATP incorporation is 2-4-fold greater in chromatin from auxin-treated

TABLE I. Ratios of [^{3}H]UTP Incorporation and [^{32}P]-α-3'dATP Incorporation into RNA in Chromatin Isolated from Untreated and Auxin-Treated Hypocotyl.

Hours after Auxin Treatment	[^{3}H]UTP Incorporated Treated/Untreated	[^{32}P]-α-3'dATP Incorporated Treated/Untreated
6	3.66 ± 1.14	1.66
12	4.05 ± 1.19	1.53 ± 0.37
24	7.45 ± 0.73	1.80 ± 0.31
48	7.91 ± 0.41	1.77 ± 0.34

Ratios are based on equivalent amounts of chromatin DNA in untreated and auxin-treated hypocotyl. Assays were for 30 minutes at 21 C. Data are presented as mean value ± 95% confidence interval.

hypocotyl compared to untreated hypocotyl (Table I), and this agrees with previous results (11).

Although we have previously demonstrated that the bulk of the RNA polymerase activity associated with chromatin prepared by the procedures employed here is RNA polymerase I (15, 16), we have conducted additional experiments which indicate that chromatin-bound RNA polymerase I is the enzyme responsible for the incorporation of [^{3}H]UTP and [^{32}P]-α-3'dATP observed in Figures 1 and 2 and Table I. First, the results summarized in Figures 1 and 2 and Table I were obtained in the presence of 1 μg/ml of α-amanitin indicating that RNA polymerase II was not contributing to the incorporation of label into RNA. Similar results were obtained in the absence of α-amanitin or in the presence of 100 μg/ml of α-amanitin (data not shown). Secondly, nearest neighbor analysis indicates that poly (A) polymerase, which is inhibited by 3'dATP (17) and may utilize 3'dATP as a substrate, does not appear to be responsible for the incorporation of [^{32}P]-α-3'dATP that we observe here (Table II). Incorp-

TABLE II. Nearest Neighbor Analysis of RNA Synthesized In Vitro with [^{32}P]-α-3'dATP as the Labeled Nucleotide.

Source of Chromatin	Fraction of Total Counts Incorporated AMP	CMP	GMP	UMP
Untreated Hypocotyl	0.19	0.20	0.35	0.25
Auxin-Treated Hypocotyl	0.19	0.17	0.40	0.24

Nearest neighbor analysis was performed on RNA synthesized in vitro which was subjected to alkaline hydrolysis as described by Coupar et al. (3).

TABLE III. Stability of [^{3}H]UTP and [^{32}P]-α-3'dATP Incorporated into RNA.

Source of Chromatin	CPM Incorporated [^{3}H]UTP 10 minute pulse	[^{3}H]UTP 60 minute chase	[^{32}P]-α-3'dATP 10 minute pulse	[^{32}P]-α-3'dATP 60 minute chase
Untreated Hypocotyl	803	817	6003	6150
Auxin-Treated Hypocotyl	6735	6728	7778	7727

Chase experiments were conducted by diluting the labeled nucleoside triphosphate 75-fold with the corresponding unlabeled nucleoside triphosphate at 10 minutes after the assays were initiated.

oration of [^{32}P]-α-3'dATP by poly (A) polymerase would have resulted in a high AMP ratio compared to other nucleotide monophosphates in nearest neighbor analysis.

We have also tested several other possibilities that could produce artifactual results. First, ribonuclease activity could lead to an underestimation of the number of elongating RNA chains if significant amounts of the incorporated [^{32}P]-α-3'dATP were enzymatically cleaved from the RNA synthesized *in vitro*. Pulse-chase experiments summarized in Table III indicate that ribonuclease does not contribute to loss of ^{32}P from *in vitro* synthesized RNA. Secondly, if initiation of RNA chains occurred *in vitro* and if RNA polymerase associated with chromatin from auxin-treated and untreated hypocotyls displayed differential rates of RNA chain initiation, the numbers of elongating RNA chains would not be accurately estimated. We have used heparin to inhibit initiation of RNA chains during the *in vitro* assay period (18, 19), and our results indicate that heparin does not significantly alter the level of [^{32}P]-

TABLE IV. The Effect of Heparin on the Incorporation of [^{32}P]-α-3'dATP into RNA in Chromatin from Untreated and Auxin-Treated Hypocotyl.

Assay Conditions	CPM Incorporated Untreated	Auxin-Treated
Minus Heparin	5531	6800
Plus Heparin	5552	6797

When present, the heparin concentration was 1 mg/ml. Assays were for 30 minutes at 21 C.

α-3'dATP incorporated. Thus initiation of RNA chains *in vitro* does not appear to occur in the chromatin system analyzed in this study (Table IV).

The method describe here may be useful for similar studies in other hormone-induced systems. This method may have wider application if another $[^{32}P]$-α-3'-deoxynucleotide triphosphate is utilized in place of $[^{32}P]$-α-3'dATP, especially in systems where poly (A) polymerase activity is abundant in chromatin or nuclear preparations.

ACKNOWLEDGMENTS

This research was supported by Public Health Service Research Grant (GM 24096).

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