CYTIDINE METHYLATION IN BUDS RELEASED FROM DORMANCY WITH 6-BENZYLAMINOPURINE

G. W. Schaeffer and F. T. Sharpe, Jr.

Crops Research Division, Agricultural Research Service,
U. S. Department of Agriculture, Beltsville, Maryland

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This paper shows that the methylation of macromolecules of tobacco buds occurs during the early phases of bud release from dormancy by the exogenous application of 6-benzylaminopurine (BAP). We also show by co-chromatography with authentic 5-methylcytidine that one of the methylated nucleosides recovered from macromolecules of stimulated buds is methylcytidine.

The cytokinin 6-benzylaminopurine breaks dormancy of inhibited buds and stimulates RNA synthesis as well as the incorporation of thymidine into macromolecules within 24 hr after BAP treatment (Schaeffer and Sharpe, 1969). This communication shows that RNA isolated from buds activated with BAP has elevated levels of methylcytidine, and that methylation precedes the increase in RNA synthesis which occurs between 19 and 24 hr after treatment with 2.5×10^{-4} M aqueous BAP, (Schaeffer and Sharpe, unpublished).

The observation that nucleobases are methylated at the polynucleotide level (Borek, et al. 1962), has focused interest on the functional and developmental significance of this base methylation. It is known that the level of nucleic acid methylation changes during sea urchin development (Comb, 1965), and during morphogenesis of the cellular slime mold (Pillinger and Borek, 1969). The knowledge that tumor tissues have high levels of methylase activities created additional interest in the function of methylation during the initiation of cell division (Tsutsui, et al., 1966). The role of t-RNA in regulation is now an area of intensive interest and the isolation of minor bases, and particularly purine derivatives

which function as plant cytokinins, (Armstrong, et al., 1969), from specific t-RNAs has broadened the scope of the work on RNA methylation.

MATERIALS AND METHODS

Axillary buds of N. tabacum cv Maryland Catterton, were stimulated with 50 µl of aqueous 2.5 x 10^{-4}M BAP in 0.1% Tween 80 applied with a syringe. At the same time the control buds received an equal volume of 0.1% Tween 80 only. BAP was applied once at the initiation of 2 treatments of either 19 or 27 hr duration. The buds were excised and exposed to the isotope for the terminal 3 hr of the treatment period. The excised buds were placed into 2 ml of solution at pH 6.0 containing 4uc/ml ^{14}C-methyl-methionine (*Tracer Labs - 13.54 mc/mM), 10^{-4}M dithiothreitol, 10^{-4}M ammoniumacetate, 200 µg of chloramphenicol (*Parke, Davis & Co.) and 0.8% sucrose. Buds were placed into the isotope solution for 1 hr at 25 C, and then placed on filter paper in petri dishes moistened with the incubation medium for an additional 2 hr. Next they were rinsed 3 times with 2.4 x 10^{-4}M non-radioactive methionine at 2C, and the nucleic acids were extracted according to the phenol-Nalaurylsulfate method of Cherry, et al., 1965. The macromolecules were dialyzed against phosphate buffer pH 6.7 overnight and then dialyzed against glass distilled water for 2 hr. The nucleic acids were precipitated with 2 volumes of ice cold absolute ETOH. The pellet was suspended once more in a small volume of water and freeze-dried for later use. Nucleic acids were exposed to bovine pancreatic ribonuclease (*Nutritional Biochemicals - 5 x crystalline) at 6 µg/ml for 3 hr at 37 C. The ribonuclease treatment was repeated. The unhydrolyzed macromolecules were precipitated with ETOH and the pellet digested with 0.3N KOH for 16 hr. The supernatant was treated with 0.3N KOH to complete the hydrolysis, then handled identically with, but separately from, the KOH soluble proportion of the pellet. The KOH treated materials were passed through *Dowex 50 and then dephosphorylated with snake venom (*Worthington Biochemical Corp.) and

alkaline phosphatase (*Worthington Biochemical Corp.) as described by Hall 1964. The nucleosides were concentrated and chromatographed on *Whatman #3 paper in a descending manner in isopropanol-NH_4OH(conc.)-H_2O (7/1/2)(v/v/v).

The paper was divided into lower, middle, and upper sections containing the following nucleosides: (typical Rf of the standards in parenthesis): Lower, guanosine (0.31); Middle, uridine (0.47), cytidine (0.53), 5-methylcytidine (0.58), adenosine (0.58); Upper, 6-methyladenosine (0.74) and 6-dimethyladenosine (0.81). Each section of the 1-dimensional paper was eluted with H_2O, concentrated in a flash evaporator, and spotted on *Whatman #1 paper for 2-dimensional chromatography, both in a descending flow. The solvent for the first dimension was n-butanol-H_2O-formic acid (77/11/12)(v/v/v), and the second was n-butanol-H_2O-NH_3 vapor (86/14)(v/v). The chromatograms were then cut into 3.0 x 3.0 cm sections and monitored for radioactivity in PPO, dimethyl-POPOP cocktail with a *Packard scintillation spectrometer.

The paper squares containing the methylcytidine were removed from the scintillation cocktail for additional chromatography. They were washed 3 times with toluene, and 3 times with chloroform. The papers were then macerated and eluted 3 times with water. The samples were then freeze-dried and later spotted on Whatman #1 paper for rechromatography in isopropanol-NH_4OH(Conc.)-H_2O (7/1/2)(v/v/v).

RESULTS AND DISCUSSION

The growth stimulation of tobacco buds is accompanied by an increase in the methylation of RNA. Buds activated with 10^{-3}M aqueous BAP showed an initial increase in the level of RNA over the control between 20 and 24 hr (Schaeffer and Sharpe, unpublished). Also, in these experiments only a small increase in the level of RNA in the BAP-treated over the controls occurred at 19 hr. The BAP-treated/control ratios for the total RNA levels were 1.28 and 1.67 for the 19 and 27 hr treatments,

respectively. The optical density data show again that RNA synthesis was submaximal at 19 hr and was more pronounced at 27 hr.

Even though the synthesis of RNA was highest at 27 hr, the rate of ^{14}C-methyl incorporation was high in the BAP-treated material at both times. The BAP-treated/control ratios of unfractionated nucleotides from KOH hydrolyzed RNA were 1.44 and 1.12 for the 19 and 27 hr treatments, respectively. This supports the concept that the methylation rate changes during development, and that the maximum rate of methylation occurs before a full complement of new RNA has been synthesized.

Additional purification of the KOH hydrolyzate by chromatography with paper resolved the nucleosides into lower, middle and upper fractions described in Materials and Methods. Illustrated in Table I are the optical density and count recovered from the three portions of the chromatograph.

Also shown in Table I is the high level of isotope incorporation into BAP-activated materials. On 2-dimensional chromatography some of the components in each fraction did not represent identified minor bases. After additional purification, the specific isotope activity per unit of cytidine recovered was at least 2 times as high at the 19 hr period as it was at 27 hr.

The methylcytidine was then removed from the scintillation vials after a 50-minute count, and rechromatographed along with authentic non-radioactive 5-methylcytidine as carrier. The count recovered from the UV absorbing spot representing 5-methylcytidine was nearly the same for the 19 and 27 hr BAP-treated materials. However, the initial level of the KOH hydrolyzed RNA, and consequently the level of cytidine recovered later from the 2-dimensional chromatograph at the 27 hr BAP-treated material, was nearly 2 times that of the 19-hr. Both controls had RNA levels similar to that of the 19-hr activated material. Thus, the specific activity from the methylation of RNA is over 2 times as high at 19 hr after activation than at 27 hr after BAP treatment (Table II).

TABLE I

Optical density and count recovered from the paper chromatograph in separation of KOH hydrolyzed RNA of BAP-treated and control buds. Digests were neutralized through Dowex 50 and the nucleotides were dephosphorylated with alkaline phosphatase and chromatographed on Whatman #3 paper in isopropanol-NH_4OH (Conc.)-H_2O (7/1/2) (v/v/v).

Treatment time (hr)	Optical Density Units Recovered from Paper 1/ (Absorbance at 260 mu)	DPM (Total recovered)
Lower Section: containing guanosine		
19 BAP	6.99	1440
19 Control	6.72	911
27 BAP	9.77	1338
27 Control	5.88	773
Middle Section: containing uridine, cytidine, adenosine, and methyl-cytidine		
19 BAP	12.29	1831
19 Control	11.87	1247
27 BAP	18.73	2731
27 Control	11.03	992
Upper Section: Rf for methyladenosine and dimethyladenosine 2/		
19 BAP	--	633
19 Control	--	386
27 BAP	--	773
27 Control	--	320

1/ The quantity of RNA extracted from equal numbers of treated and control buds at the two time periods was: 19 BAP - 1.18 mg; 19 Control - 1.14 mg; 27 BAP - 2.16 mg; and 27 control - 1.08 mg.

2/ No UV absorbing bands visible.

The recovery of methylcytidine from macromolecules during early phases of bud activation with BAP shows that methylation of RNA is one of the initial reactions in nucleic acid metabolism, and implies some coupling with regulatory systems. This phase of active methylation precedes or occurs concomitantly with the initial stages of new RNA synthesis.

TABLE II

DPM recovered by co-chromatography of authentic 5-methylcytidine with ^{14}C-methylcytidine isolated from RNA partially purified by 3 previous solvent partitions on paper. The solvent for this final chromatography was isopropanol-NH_4OH(Conc.)-H_2O (7/1/2)(v/v/v).

Treatment time (hr)	DPM	Micromoles of cytidine 1/ recovered from 2-dimensional chromatography	DPM/micromole cytidine	Ratio BAP/control
19 BAP	247	0.23	1074	3.76
19 Control	80	0.28	286	
27 BAP	251	0.56	448	1.65
27 Control	65	0.24	271	

1/ This refers to the cytidine recovered from the previous 2-dimensional chromatography.

In another set of experiments budsactivated for 12 and 27 hr were combined for extraction after incubation with [^{14}C-methyl]S-adenosyl-L-methionine (*New England Nuclear - 55 mc/mM) as methyl donor. The counts recovered from chromatographic positions representing methylcytidine were 3 times as high in the BAP-activated tissues as in the controls. That is, the average radioactivity due to methylcytidine in BAP-treated buds was 2303 DPM per micromole of cytidine recovered from 2-dimensional paper chromatograph of KOH hydrolyzed RNA representing both time periods. The level for control buds was 741 DPM per micromole of cytidine recovered and the BAP-treated/control ratio of 3.1 agrees well with the data reported here.

Two portions of these experiments warrant some emphasis. (1) The use of chloramphenicol in the incubation medium to prevent microbial growth probably prevents the O-methylation of some ribosides, but chloramphenicol does not prevent the initial bud activation with BAP (Schaeffer and Sharpe, 1969), as measured by thymidine incorporation into macromolecules. (2) The specific activity in the ribonuclease resistant fractions of the bud nucleic acid is higher than the specific isotope

activity observed in the RNAse hydrolyzed fraction. The latter response suggests that there is a close relationship between the resistance of RNA to ribonuclease (Srivastava, 1968), and the methylation of nucleic acids during cytokinin activation.

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