Notes

RIBONUCLEASES CATALYZE THE SYNTHESIS OF B-FACTOR (3'-BUTYLPHOSPHORYL AMP), AN INDUCER OF RIFAMYCIN PRODUCTION IN A *NOCARDIA* SP.

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B-Factor, 3'-(1-butylphosphoryl)adenosine, is an inducer of rifamycin B production in a rifamycin non-producing *Nocardia* mutant derived from its parental strain KB-993. This factor was discovered first in yeast extract (Difco)[1] and then also in the parental *Nocardia* strain[2]. Addition of B-factor (10^{-8} M) induces production of rifamycin B in the mutant, as do B-factor analogues, *i.e.*, alkylesters of 3'-AMP with alkyl side chains of C(2)~C(12), and *n*-butyl esters of 3'-GMP and 2'-AMP[2]. In this report, we show that B-factor is synthesized from butanol and either 2',3'-cAMP or RNA by the action of RNases.

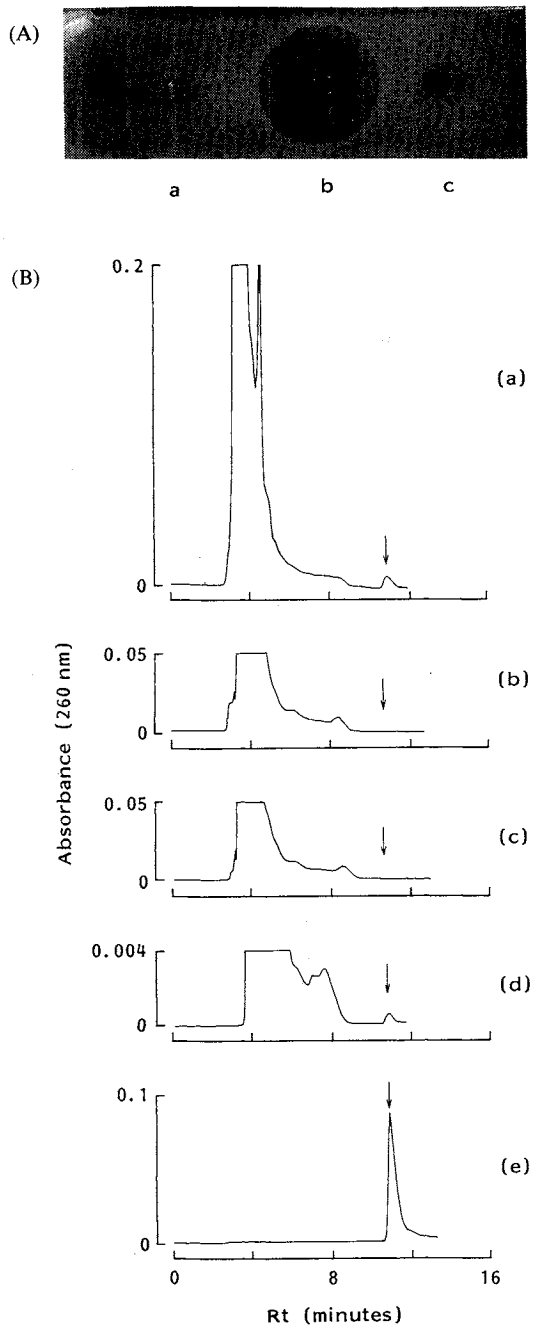
A rifamycin non-producing *Nocardia* mutant derived from strain KB-993 was used for bioassay of B-factor as described previously[1]. *Saccharomyces uvarum* IFO 0751 (beer yeast) was cultured in the medium containing malt extract (Difco) 2%, Polypepton (Wako) 0.1% and glucose 2%, pH 7.0, at 30°C without shaking. *Nocardia* KB-993 was cultured in medium containing Nutrient Broth (Difco) 0.8%, glucose 0.5% and NaCl 0.3%, pH 7.0, at 30°C with shaking. The cells were harvested by centrifugation, washed once with 50 mM phosphate buffer, pH 7.0, and 1 g of the wet cells, disrupted by grinding with 1 g of aluminum oxide, was suspended in 1 ml of phosphate buffer, pH 7.0. The cell extracts recovered by centrifugation at 7,000 × *g* for 10 minutes were examined for *in vitro* synthesis of B-factor. The reaction mixtures were analyzed by reverse phase HPLC equipped with a Senshu SSC-ODS-1251 column using a solvent system composed of CH_3NO-0.1 M citrate buffer (15:85), pH 5.0.

In order to detect the B-factor synthesizing activity in the cell extracts, we examined 3'-AMP, 3',5'-cAMP and 2',3'-cAMP as possible substrates to be esterified with butanol. The reaction mixtures containing 3.7 μmol of one of these nucleotides, 3.5 μmol of butanol and 15 μl of cell lysate in a total volume of 75 μl of 50 mM phosphate buffer, pH 7.0, were incubated at 30°C for 12 hours, and then analyzed by HPLC. As shown in Fig. 1(B), a small but distinct peak exactly coinciding with authentic B-factor was detected with the cell extract of *S. uvarum* incubated with 2',3'-cAMP and butanol. No such peak was detected in the reaction mixtures containing 3'-AMP or 3',5'-cAMP. Bioassay also confirmed that B-factor was produced from 2',3'-cAMP but not from 3'-AMP nor 3',5'-cAMP (Fig. 1(A)). Approximately 0.01 μmol of B-factor was produced from 3.7 μmol of 2',3'-cAMP by the *S. uvarum* extract during 4 hours incubation. The same B-factor synthesizing activity was detected by HPLC in extracts of *Nocardia* strain KB-993 and *Schizosaccharomyces pombe* 972h$^-$ but not in extracts of *Aspergillus oryzae* IAM 2670 and *Escherichia coli* C600.

Since RNases are known to catalyze transesterification *via* 2',3'-cAMP as a reaction intermediate[3], we examined B-factor synthesis by RNases from various origins. RNase A from bovine pancreas (Sigma, 5,000 U), RNases T1 and T2 from *A. oryzae* (Sigma, 5,000 and 500 U, respectively), RNase U2 from *Ustilago sphaerogena* (Sigma, 500 U) and partially purified RNase mixture from *Aspergillus clavatus* (Sigma, 5,000 U) were added to reaction mixtures as above in place of the cell extract and incubated with 2',3'-cAMP and butanol. As shown in Table 1, synthesis of B-factor was detected with RNases T1 and U2 and the partially purified RNase mixture from *A. clavatus* but not with RNases A and T2. When 2 mg of yeast RNA (Sigma) was added to the reaction mixture in place of 2',3'-cAMP, again synthesis of B-factor was detected with the extract of *S. uvarum* (Fig. 1(B)) and RNases of *A. clavatus* (data not shown). These results clearly indicate that B-factor can be produced during digestion of RNA by some RNases in the presence of butanol.

It was shown that RNases A, T1, U2 and the partially purified RNase mixture from *A. clavatus* could catalyze the synthesis of dinucleoside monophosphate from nucleoside-2',3'-cyclic phosphate and a free nucleoside[4~7]. However, with

Fig. 1. Detection by HPLC of B-factor synthesized *in vitro* using 2',3'-cAMP and butanol as the substrates and the cell extract of *Saccharomyces uvarum*.



The reaction mixture was mixed with an equal volume of ethanol and then cooled −80°C for more than 5 minutes. After centrifugation at 7,000 × *g* for 5 minutes, the upper phase was applied to HPLC and paper disc for plate assay.

(A) Plate assay of B-factor. A portion of the frozen cell suspension of the rifamycin B non-producing mutant was mixed with 100-fold volume of nutrient soft agar solution (agar 0.5%) at a temperature as low as possible. This mixture (10 ml) was overlayed on 50 ml production medium base plate (agar 2%). A paper disc containing a test sample was placed onto the agar solution (agar 1.0%) containing spores of *Bacillus subtilis* was overlayed. The plate was incubated at 37°C for 1 day and then an inhibitory zone was observed[1].

Paper disc contains a: none, b: the complete reaction mixture incubated for 12 hours, c: the complete reaction mixture without incubation, as a negative control.

(B) HPLC assay of B-factor. B-Factor synthesized was detected by measuring absorbance at 260 nm. a: The complete reaction mixture incubated for 12 hours, b: the complete reaction mixture without incubation, as a negative control, c: the reaction mixture minus the cell extract, as a negative control, d: yeast RNA was used in place of 2',3'-cAMP, e: authentic B-factor, as a control.

Table 1. *In vitro* synthesis of B-factor with various RNases by using 2',3'-cAMP and butanol as the substrates.

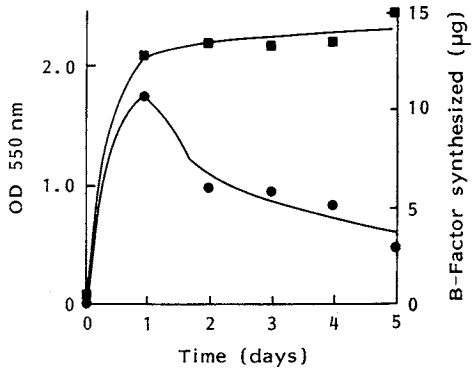
RNases	Scission specificity	RNase activity (U)	B-Factor synthesized (nmol)
RNase T1	G	5,000	0.09
RNase U2	A, G	500	0.16
RNase A	C, U	5,000	0
RNase T2	A, G, C, U	500	0
Partially purified RNase of *Aspergillus clavatus*	A, G, C, U	5,000	11
Lysate of *Saccharomyces uvarum*	—	—	2.3

B-Factor synthesized in the reaction mixture for 4 hours was calculated from the areas obtained by integration of the peak having the same Rt as that of B-factor.

RNases A, T1 and U2, the synthesis was restricted to substrates with specific base sequences. The difference in activities of these RNases catalyzing 2',3'-cAMP and butanol may be due to their specificity for adenine nucleotide. A relatively high activity to synthesize B-factor was observed with

Fig. 2. Time course of B-factor synthesizing activity of the cell lysate of *Saccharomyces uvarum* when 2',3'-cAMP and butanol were used as the substrates.

■ Growth (OD 550 nm), ● B-factor synthesized in the 75 μl reaction mixture for 4 hours was calculated from the peak area having the same Rt as that of B-factor.



RNase U2 which catalyzes transesterification of purine nucleoside 2',3'-cyclic phosphate, while no activity was detected with RNase A which recognizes pyrimidine nucleoside 2',3'-cyclic phosphate. The activity with an excess amount of RNase T1 which primarily recognizes guanosine 2',3'-cyclic phosphate may be due to the lower specificity for adenine nucleotides in this synthesis reaction. Although these observations support the idea that B-factor is synthesized by typical RNases recognizing purine nucleoside 2',3'-cyclic phosphate, we can not rule out that the strong activity observed with the RNase preparation from *A. clavatus* may be due to that some other enzyme with high transesterification activity may exist in the preparation. On the other hand, specific activity to synthesize B-factor in the cell extract of *S. uvarum* showed a characteristic change during the exponential to the stationary growth phases (Fig. 2). Further experiments are necessary to identify the enzyme responsible for B-factor synthesis in yeast.

We may assume that nucleotides of the B-factor type play a regulatory role for cellular function not only in *Nocardia* but also in other microorganisms in general.

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