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DISTRIBUTION OF OLIGONUCLEOTIDE PRODUCTS DURING INHIBITION OF THE RNA POLYMERASE-CATALYZED SYNTHESIS OF RNA BY DAUNOMYCIN

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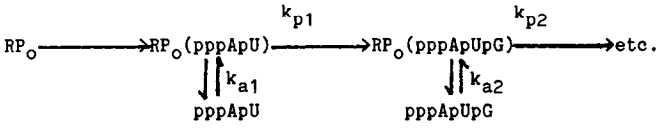
The anticancer agent daunomycin (dau) has been shown to inhibit the synthesis of RNA both in cultures of mammalian cells (1) and in vitro (2). Using the strong A promoter of T7 phage, in a system consisting of E. coli RNA polymerase and T7 DNA template, we have previously shown that dau does not interfere with the formation of a specific enzyme-promoter complex, until the template is saturated by dau molecules. Instead, indirect evidence suggested that dau interferes with the incorporation of some of the first few nucleotides into initiated RNA chains (3). More recent results also suggested that dau has little effect on the substeps leading to the formation of abortive dinucleotide from the A promoters. The major inhibitory effect of dau was found to be specifically exerted during the transformation of the initial dinucleotide to the corresponding trinucleotide (4). In this report we examine the effect of dau on the time-course of the RNA polymerase-catalyzed synthesis of di-, tri-, tetra- and pentanucleotides synthesized from the A3 promoter of T7.

MATERIALS AND METHODS: Oligonucleotide Synthesis-A mixture of di-, tri-, tetra-, penta-, and hexanucleotides was synthesized from the A3 promoter using T7 DNA (200 μM final concentration) and RNA polymerase (0.2 μg) in buffer (40 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 10 mM 2-mercaptoethanol). Buffer (5 μl, 1mM) at a 5-fold higher concentration was added into T7 DNA (5 μl, 1 mM), followed by a solution of the enzyme (5 μl). Daunomycin (5 μl of appropriate concentrations) or water (5 μl) was added, and the solution was preincubated for 10 min at 37°C. Finally, a mixture of ATP (5 μl, 500 μM), [α-^{32}P]UTP (50 μM; specific activity, 2,650 cpm/pmol, and GTP (25 μM) was added, and the solution was incubated for various intervals at 37°C. For the 5 min incubation, the added mixture was varied, e.g. by omitting GTP or by using [α-^{32}P]GTP (25 μM; specific activity, 3,350 cpm/pmol) instead of labeled UTP, in order to correlate specific electrophoresis bands with oligonucleotide size as previously described (4). The reaction was interrupted with the addition of urea (5 μl, 8M) and buffer (10 μl) consisting of 150 mM Tris borate, pH 8.0, 3mM EDTA, 50% glycerol, 0.01% bromphenol blue, and 0.01% xylene cyanol. Acrylamide Electrophoresis-An aliquot (25 μl) of the mixture described above was electrophoresed on 24% acrylamide (1:29 ratio of acrylamide to methylenebisacrylamide) gel plates (0.20 x 20 x 40 cm) for 16 h at 200 V (5 mA) in 7 M urea, 50 mM Tris Borate, pH 8.00, and 1 mM EDTA. Identification and quantitation of products was carried out as previously described (4).

RESULTS AND DISCUSSION: The formation of oligonucleotide products during the late stages of RNA chain initiation from the A3 promoter may be described by the following general scheme:



where k_{a1}, k_{a2}...represent constants for the dissociation of oligonucleotides from the ternary enzyme-template-oligonucleotide complexes and k_{p1}, k_{p2}...represent composite constants for the combined nucleotide addition-translocation process leading to the elongation of RNA chains. RNA polymerase recycles over the promoter (prior to the formation of a stable ternary complex) producing

oligonucleotides reiteratively (4).

Under the experimental conditions used in this study, the only oligonucleotide products that are detectable originate from the A1 promoter and the A3 promoter (4); the latter giving a product with the starting sequence pppApUpGpApApA. Correction for the concurrent synthesis of pppApU, the sole product from the A1 promoter, is made using the D111 T7 DNA mutant, which lacks the A3 promoter, as template. Information regarding the mechanism of inhibition of RNA synthesis can be obtained from the distribution of oligonucleotide products, as dau concentrations increase. The distributions, obtained for transcriptions intervals between 1 and 7 min in the absence of dau and in the presence of an added dau to DNA (base pair equivalent) molar ratio of 0.13, are shown in Fig.1

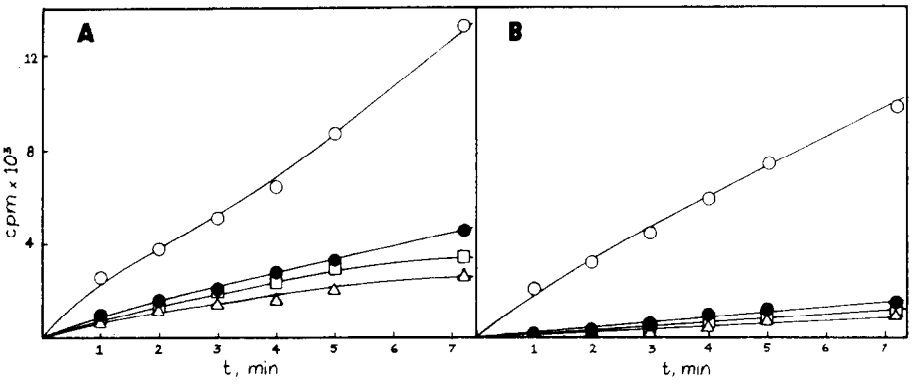


Fig. 1. Distribution of oligonucleotide products for various transcription reaction times in the absence of dau (A); and in the presence of a added dau to DNA ratio of 0.13 (B): —○— di-; —●— tri-; —△— tetra-; and —□— pentanucleotide as measured by [α-^{32}P]UTP incorporation. Results for reactions over 5 min may be influenced by reinitiation(4).

These and additional data (not shown) obtained at ratios of 0.05 and 0.107 indicate that up to a ratio of 0.107 the rate of dinucleotide synthesis, as calculated from the quantitation of product after 5 min of transcription, remains fairly constant at over 90% of the uninhibited rate. However the rate of synthesis of longer oligonucleotides is decreasing progressively to less than 50% of the uninhibited rate. Also at the 0.107 and 0.13 ratios tri-, tetra-, and pentanucleotides collectively decrease to about 30% of total product, as compared to about 45% of product in the absence of dau.

The results obtained with dau concentrations below the level of template saturation, indicate that the inhibitor interferes with the formation of trinucleotide without affecting the stability of the RPo(pppApU) complex (ie the k_{a1} constant). We therefore postulate that dau interferes primarily with the transformation of RPo(pppApU) to RPo(pppApUpG). The inhibitor has a very small, if any, affect on k_{p2}, k_{p3} or k_{p4}. Alternatively dau may have no effect on the recycling of the enzyme from the dinucleotide level but it may interfere with recycling from the trinucleotide level, thus leading to decreased reiterative synthesis of oligonucleotides that are longer than dinucleotide. We conclude that the behavior of dau is distinct from that of other inhibitors that are known to act at the same late stage of transcription initiation; namely α-aminitin, which stabilizes the ternary complex, (5) and rifampicin, which appears to lead to increased acculumation of the ternary complex (6).

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