IN VITRO INHIBITION OF E. COLI RNA POLYMERASE TRANSCRIPTION OF RAT LIVER CHROMATIN BY AFLATOXIN B_1*

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Aflatoxin B_1, a potent hepatocarcinogen produced by the mold Aspergillus flavus, is known to produce, after injection, a number of diverse biochemical effects (1). These include a strong inhibition of RNA synthesis (2-4). In vitro experiments have shown that aflatoxin can interact with DNA, which has led to speculation that the inhibitory effect of aflatoxin in RNA synthesis might result from its binding to the DNA template, in a manner similar to actinomycin D (3). However, because of a failure to produce this inhibition of RNA synthesis in vitro, other workers have proposed that a metabolite of aflatoxin might be the causative agent (5, 6). Roy (6) found that aflatoxin B_1 did not inhibit an in vitro RNA synthesizing system containing calf thymus DNA and rat-testicular RNA polymerase; also, King and Nicholson (7) were unable to obtain in vitro inhibition using E. coli RNA polymerase and calf thymus DNA using the usual concentrations of cations in the assay system. These latter workers did obtain a very slight inhibition (11-13%) of questionable significance when Mg^{++} was omitted and Mn^{++} was

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kept at a suboptimal concentration (0.5 mM). Wogan (8), in a review of these reports, has recently concluded that, for the most part, the results have been negative; i.e. DNA-aflatoxin complexes are transcribed as effectively as DNA alone.

On the other hand, when rat liver chromatin is used as template, rather than pure DNA, we have found that the in vitro inhibition of RNA synthesis reaches 78%, thereby achieving the reported in vivo inhibition as well as demonstrating the significance of the non-nucleotide components of chromatin in the interaction with aflatoxin.

METHODS

Aflatoxin B_1 was purified by the method of Hanna and Campbell (9). The purity was checked by its UV spectrum and by its ability to migrate on thin layer chromatograms as a single spot in at least two different solvent systems. In all experiments, 200 gram Sprague Dawley-derived male rats were used. Chromatin was isolated by the method of Marushige and Bonner (10) and DNA by the method of Church and McCarthy (11).

All template activity studies were incubated at 37^{o} in a total volume of 0.25 ml containing 10 μmoles of Tris·HCl (pH 8.0), 1.0 μmole $MgCl_2$, 3.0 μmoles mercaptoethanol, 0.1 μmole of UTP, ATP, CTP, and GTP (all nucleoside triphosphates were purchased from Schwarz Bio Research), 3 units of RNA polymerase (Miles Laboratories), 2.5 μCi ^{3}H-UTP (Schwarz Bio Research, spec. act. 17.3 Ci/mmole) and varying amounts of rat liver DNA or chromatin. After the addition of aflatoxin B_1 in 0.02 ml of dimethylformamide, the assay mixture was preincubated for 5 minutes. The reaction remains linear for 10 minutes and was initiated by the addition of RNA polymerase. The incorporation of ^{3}H-UTP into RNA was measured by the procedure of Mans and Novelli (12). After 10 minutes of incubation, 100 μl of the

incubation mixture was removed and rapidly applied to a filter disc (Whatman 3MM). The disc was quickly dried and washed three times in 10% TCA containing 2 mM sodium pyrophosphate. The disc was then washed in ethanol-ether (1:1) to remove any traces of water and counted in 10 ml of PPO-POPOP toluene counting cocktail in a Beckman LS-133 spectrometer. DNA was determined by the modified procedure of Burton (13).

RESULTS AND DISCUSSION

As can be seen in Figure 1 aflatoxin only slightly inhibits RNA synthesis (approximately 5%) when a highly purified preparation of rat liver DNA is used as a

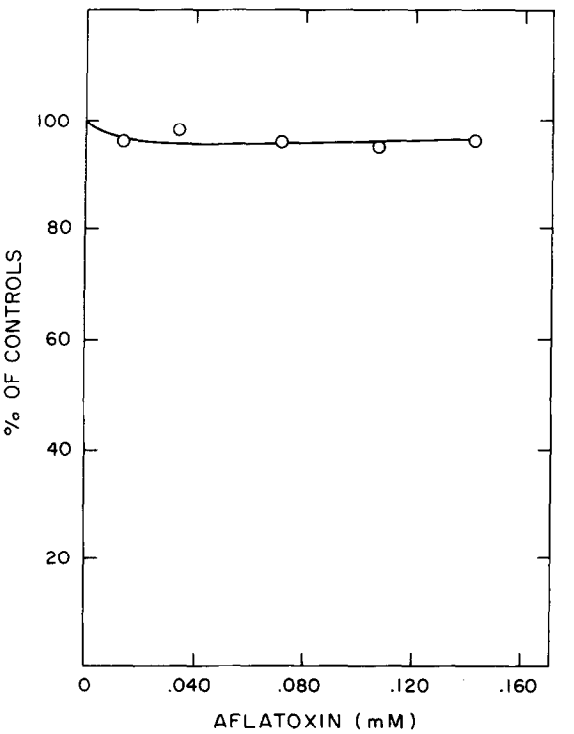

Figure 1. Template activity of rat liver DNA versus aflatoxin B_1 concentration. The concentration of DNA (expressed in terms of nucleotide monomers with an average molecular weight of 350) was 0.069 mM and the incubation volume was 0.25 ml. Incorporation of ^{3}H-UTP was measured in the aflatoxin-treated system and expressed as a percentage of control (no aflatoxin). After five minutes of preincubation, E. coli RNA polymerase was added to initiate the reaction which was allowed to proceed for 10 minutes. All concentrations were run in duplicate.

template. These results are in general accord with those of Roy (6) and King and Nicholson (7). Inhibition is greatly increased, however, when sheared chromatin rather than DNA is used as the template. Figure 2 represents two different experiments using different concentrations of chromatin DNA (0.034 and 0.068 mM, calculated using 350 as an average molecular weight for nucleotides). From this figure it can be seen that the in vitro addition of aflatoxin can produce 78% inhibition of RNA synthesis when compared with control values. Furthermore, when a constant concentration of aflatoxin (0.072 mM) was tested with increasing amounts of chromatin DNA (Figure 3) the degree of inhibition decreased, indicating the dilution of

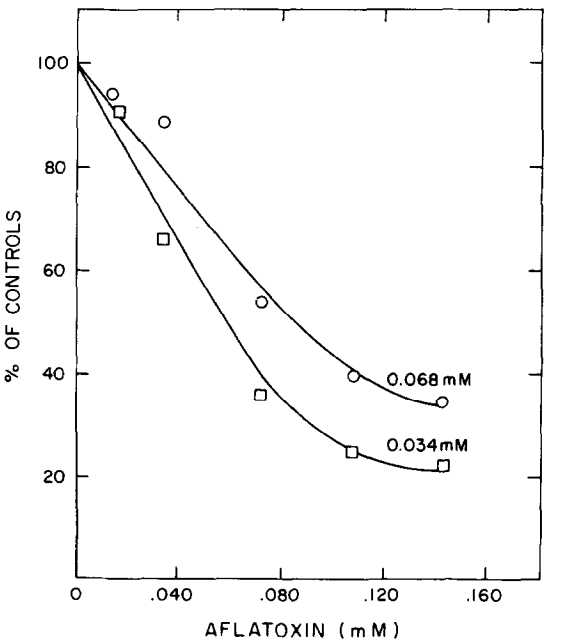

Figure 2. Template activity of constant concentrations of rat liver chromatin versus aflatoxin B_1 concentrations. Conditions of assay were the same as shown in Figure 1 and the text, except for the concentrations of chromatin DNA (0.034 and 0.068 mM) and aflatoxin B_1.

aflatoxin with DNA.

The interaction of chemical carcinogens with chromatin has been studied by others (14, 15). These groups have shown that chemical carcinogens are able to

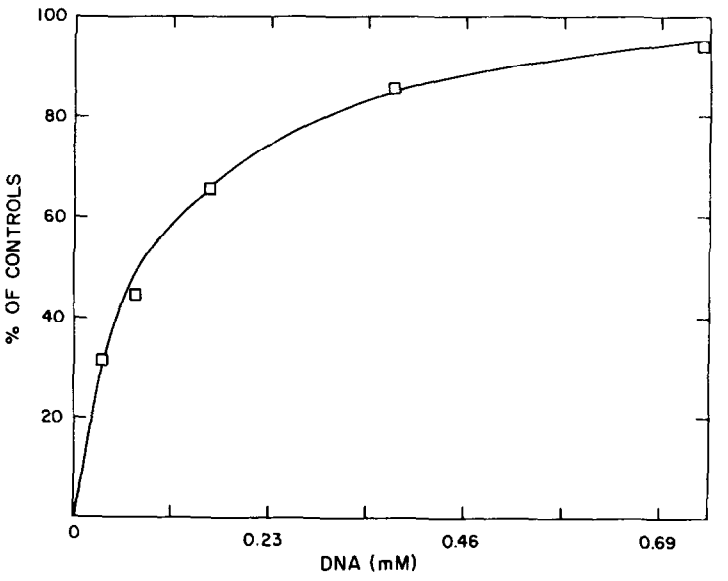

Figure 3. Template activity of varied concentrations of rat liver chromatin versus a constant concentration of aflatoxin B_1 .072 mM. Conditions of assay same as in Figure 1 and text, except for concentrations of DNA and aflatoxin.

bind in vitro to DNA and nucleohistone particles. O'Brien and co-workers (16) have also reported that both methylcholanthrene and benzpyrene can bind to chromatin in vivo. Lending credence to a possible aflatoxin-chromatin interaction is a previous report by Black and Jirgensons (17) who demonstrated by equilibrium dialysis that aflatoxin can bind to two highly purified lysine-rich histone fractions. Further evidence for the role of histone protein is the report of Moule and Fraysinet (5) who found that the template activity of rat liver DNP eliminated of histones by saline extraction also was unaffected by the in vitro addition of aflatoxin.

Of course it might be noted that studies with polycyclic hydrocarbons may not be completely analogous to our studies with aflatoxin since most of these compounds are known to increase RNA synthesis (18) whereas aflatoxin is a strong inhibitor of macromolecular biosynthesis. But in spite of this dissimilarity it should be recognized that these compounds can interact with chromatin and result in changes in its

transcriptional activity. Since DNA in mammalian cells is normally associated with histone protein these experiments may better simulate the physiological condition. Also, since microsomal enzyme activity was not present in these incubations metabolic conversion of aflatoxin B_1 to the hydroxylated derivative of M_1 may not be essential in the inhibition of DNA-directed RNA synthesis, as suggested by others (5, 6).

Sporn et al. (4) found that one mole of aflatoxin B_1 was bound to 600 moles of native calf-thymus DNA-phosphorus in equilibrium dialysis experiments whereas Wogan (8) recently reported that one mole of any of several aflatoxin compounds is required to saturate 25 moles of nucleotide. In our experiments (Figure 2), a ratio of aflatoxin/DNA at least 0.5 or higher is required in order to obtain a significant reduction in RNA synthesis. This would suggest a minimal effect of the previously reported aflatoxin/DNA interaction upon the subsequent template activity of DNA and therefore indicates the significance of the histone protein in the binding of aflatoxin (17) and the subsequent activity of the chromatin/aflatoxin complex.

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