STIMULATION OF *IN VITRO* TRANSCRIPTION BY RIBONUCLEASE H (HYBRIDASE)

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1 Introduction

Ribonuclease H or hybridase, first discovered by Stein and Hausen [1, 2] in thymus extracts, is an enzyme which digests the RNA moiety of DNA/RNA hybrids. We have recently detected hybridase activity in rat liver extracts [3] as well as in the integument of blowfly larvae [4] and have proceeded to purify the enzyme from rat liver cytosol [3]. The existence of the hybridase raises the question of the biological significance of the enzyme as well as of its substrate, the DNA/RNA hybrids. Such hybrids are formed during *in vitro* transcription by the eukaryotic RNA polymerase B (see below) and can also be detected *in vivo* [5, 6]. We have therefore studied the effects of hybridase on transcription on DNA and chromatin template by different RNA polymerases as well as on transcription by isolated chromatin. We have observed a stimulatory effect of the hybridase on transcription by rat liver RNA polymerase B of native rat liver DNA and of chromatin. These results are presented below.

2. Material and methods

Male Wistar BR II rats weighing between 120–140 g were used throughout. ^{3}H-UTP (1 Ci/mmole) was obtained from the Radiochemical Centre, Amersham; ATP, UTP, CTP, GTP, creatine phosphate and creatine phosphokinase from Boehringer, Mannheim; and pancreatic ribonuclease from Serva, Heidelberg.

The method for the *preparation of hybridase* will be published in detail elsewhere (W. Roewekamp, W. Schmid, B. Benecke and C.E. Sekeris, in preparation). Rat liver cytosol is submitted to chromatography on DEAE-cellulose, hydroxylapatite, phosphocellulose and Sephadex G-200. Chromatography on phosphocellulose leads to the appearance of two active fractions one eluting at 0.15 M NH_4Cl (peak A) and one at 0.25 M NH_4Cl (peak B). In the experiments presented here only peak A was used.

Preparation of rat liver DNA-dependent RNA polymerase A and B was performed according to Seifart et al. [7], the *E.coli enzyme* was prepared according to Zillig et al. [8].

The preparation of rat liver nuclei was performed by a modified Chauveau procedure [9] as described in [10].

Rat liver DNA was prepared from purified rat liver nuclei according to Doenecke and Sekeris [11], *chromatin* according to Beato et al. [12].

The RNA synthesizing system was as described in [13]. Transcription with rat liver RNA polymerase A was performed in the presence of 3.3 mM Mn^{2+} in the presence of ammonium sulphate (0.2 ionic strength) or in its absence, that of polymerase B in the presence of 3.3 mM Mn^{2+} and 0.2 ionic strength ammonium sulphate. The experiments with the *E.coli* enzyme were performed in the presence of 20 mM Mg^{2+} and 0.2 ionic strength ammonium sulphate.

The amount of DNA/RNA hybrids present in the incubates during transcription in the presence or absence of the hybridase was quantitated as follows: To aliquots of the incubation mixtures 6 γ/ml α-amanitin and/or 6 mg/ml AFO13 was added to stop RNA synthesis. The aliquots were then further incubated for 30 min in the presence of 0.6 μg/ml pancreatic ribonuclease. This concentration is enough to digest the free RNA but does not attack RNA hybridized to RNA. The amount of acid precipitable activity

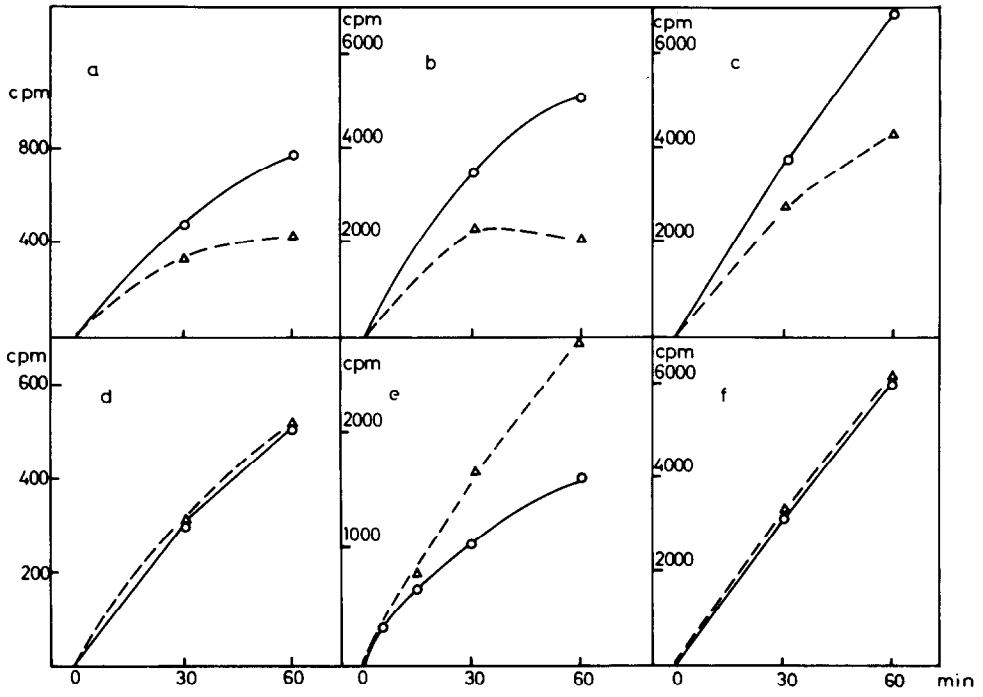

Fig. 1. Effect of hybridase on transcription of heat denatured (a, b, c) and native rat liver DNA (d, e, f) by rat liver RNA polymerase A (a, d), B (b, e) and the *E.coli* (c, f) enzyme. For experimental details see Methods. (○—○—○): Control; (△---△---△) + hybridase.

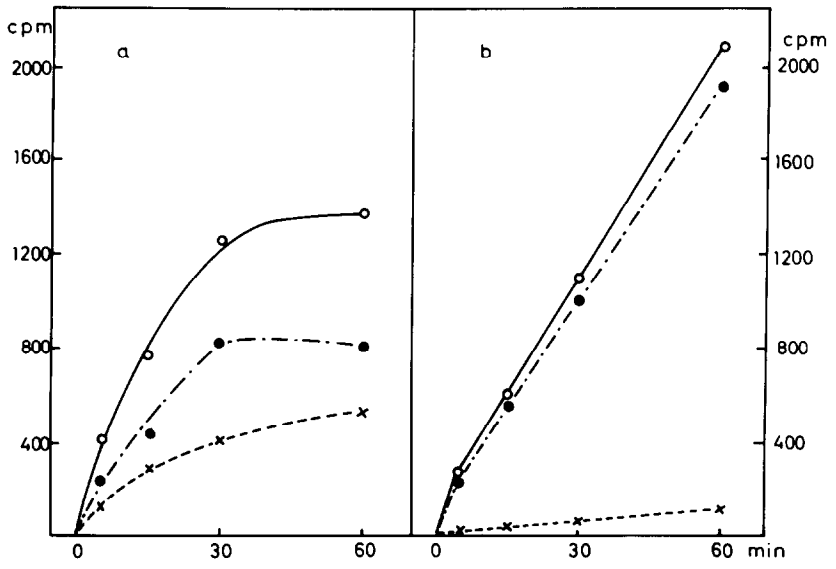

Fig. 2. Effect of hybridase on transcription of native DNA by polymerase B. For experimental details see Methods.
a) Without hybridase: (○—○—○) total RNA synthesized; (●–·–●–·–●) RNAase sensitive RNA; (x–x–x) RNAase resistant RNA.
b) In the presence of hybridase (120 μg/ml): (○—○—○) total RNA synthesized; (●–·–●–·–●) RNAase sensitive RNA; (x--x--x) RNAase resistant RNA.

remaining was measured on filter paper discs as described previously [14].

3. Results

The effect of hybridase on transcription using native and denatured rat liver DNA as template and as enzyme rat liver RNA polymerase A and B as well as *E.coli* RNA polymerase is shown in fig. 1.

Transcription on denatured DNA in the presence of hybridase is significantly impaired with all of the enzymes used. Under these conditions a considerable amount of the RNA synthesized is in form of DNA/RNA hybrids and therefore is amenable to digestion by the hybridase. The effects of hybridase on transcription with native DNA as template depend on the type of enzyme used. No effect is seen using rat liver RNA polymerase A or the *E.coli* enzyme. However, with the rat liver B enzyme a significant stimulation of transcription is observed reaching values of over 100%. The stimulatory effect is clearly evident after the first 30 min of transcription at which time period RNA synthesis in the control incubates tends to level off. In order to gain insight on the mechanism of the stimulatory effect we have titrated the amount of DNA/RNA hybrids during transcription in the presence and absence of the hybridase. This was accomplished by stopping transcription in aliquots of the incubation mixture at different time periods by adding AFO13 and α-amanitin [15] and then assaying the RNAase sensitive (free RNA) and RNAase resistant (hybrids) radioactivity. In preliminary experiments we determined the dose of RNAase (0.6 μg/ml) required for digestion of the free RNA which however leaves the hybrids intact. The results are shown in fig. 2. Transcription on native DNA in the absence of hybridase leads to the formation of substantial amounts of DNA/RNA hybrids. The amount of RNAase sensitive RNA synthesized reaches a plateau after 30 min whereas hybrids are continued to be synthesized. In the presence of hybridase almost all of the synthesized RNA is RNAase sensitive.

A similar stimulation of transcription by hybridase is also observed with chromatin preparations. Transcription by isolated nucleoli is not affected by the presence of hybridase.

In a further series of experiments we have used

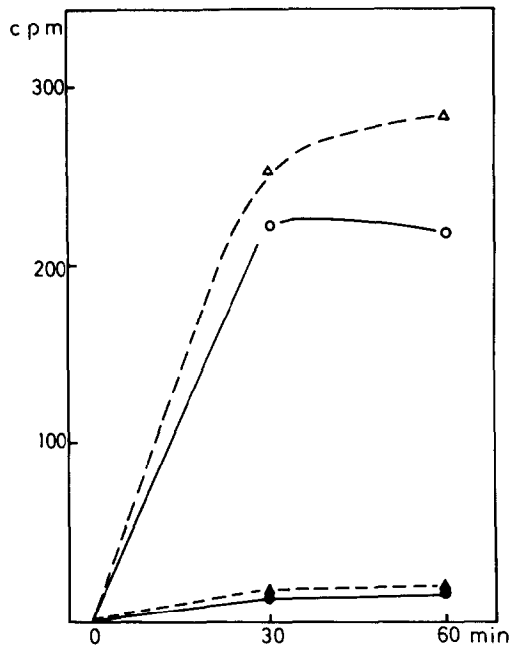

Fig. 3. Effect of hybridase on transcription of chromatin by RNA polymerase A and B. RNA polymerase A and B were incubated in the presence of chromatin (1.30 μg/DNA/ml) in a standard RNA synthesizing mixture as described in Methods. Measurement of incorporated radioactivity as in [13].
(○—○—○) Transcription by polymerase B;
(△-- △-- △) Transcription by polymerase B + hybridase;
(●—●—●) Transcription by polymerase A;
(▲-- ▲-- ▲) Transcription by polymerase A + hybridase.

chromatin as a template for transcription with added rat liver RNA polymerase A and B as well as with the *E.coli* enzyme. In the presence of hybridase transcription with polymerase B is enhanced, whereas no effect is seen with the *E.coli* enzyme (fig. 3). As previously demonstrated [16, 17] polymerase A does not effectively transcribe chromatin.

4. Discussion

The results presented above demonstrate that during transcription *in vitro* by rat liver chromatin preparations as well as by rat liver RNA polymerase B on DNA and chromatin template a significant amount of DNA/RNA hybrids are formed, depending on the conditions of the experiment. Such hybrids are

Table 1
DNA/RNA hybrids formed during transcription by isolated rat liver nuclei.

	DNA/RNA hybrids (dpm)	Total RNA synthesized (dpm)
Nuclei alone	225	2400
Nuclei + 6 γ/ml α-amanitin	60	1545
Nuclei + 120 γ/ml hybridase	40	2640

Nuclei (1.2 mg protein) were incubated in a standard RNA synthesizing mixture (see Methods) in the presence of 3.3 mM Mn^{2+}. Aliquots were removed after 10 min and the amount of total RNA synthesized and of the DNA/RNA hybrids formed measured as described in Methods.

also formed during transcription by isolated rat liver nuclei and can be also isolated from nuclei of *in vivo* pulse labelled rats [6]. In the presence of α-amanitin (see table 1) which as known inhibits DNA-like RNA synthesis [15] almost no DNA/RNA hybrids are formed *in vitro* in isolated nuclei. The general picture which emerges from the experiments in which hybridase was present during transcription is that the nuclease significantly stimulates RNA synthesis catalyzed by polymerase B and that this effect is due to the elimination of the DNA/RNA hybrids formed. This is evident in the experiments with native DNA and polymerase B, where we have titrated the amount of hybrids formed during transcription. In the absence of hybridase there is a continuous synthesis of hybrids whereas the synthesis of RNAase sensitive RNA reaches a plateau after 30 min. At that time period the overall synthesis of RNA tends to level off. In the presence of hybridase RNA synthesis becomes linear, and DNA/RNA hybrids are detected only in very low amounts, almost all of the RNA synthesized being sensitive to RNAase. It seems therefore that the hybrids formed during transcription exert an inhibitory effect on RNA synthesis and have to be removed in order that transcription can continue. This is precisely the biological role we ascribe to the hybridase. We are currently examining what step of the transcription process is affected by the hybridase.

It is interesting to note that transcription by polymerase A or the *E.coli* enzyme is not affected by hybridase. The experiments with isolated nuclei and nucleoli also support this finding. Transcription on denatured DNA is inhibited by hybridase independent of the enzyme used. Under such conditions the bulk of the RNA synthesized is in the form of DNA/RNA hybrids. A biological role of DNA/RNA hybrids was put forward by Frenster [18] and later by Britten and Davidson [19]. The authors postulated that such hybrids have a stimulatory effect on transcription. Our work tends to support the reverse, i.e. that hybrids restrict transcription. One of the roles of the hybridase then is to eliminate the formed hybrids and thus stimulate transcription.

In accord with this concept are recent findings that cortisol, which as known, stimulates RNA synthesis in the liver, leads to a decrease of *in vivo* formed DNA/RNA hybrids [6] and also to an activation of the hybridase present in rat liver nuclei [6]. Another steroid hormone, ecdysone, which stimulates transcription in the integument of blowfly larvae, also increases hybridase activity in this tissue [4]. The exact mechanism of inhibition of RNA synthesis by the DNA/RNA hybrids and the role of hybridase on transcription is currently being pursued in our laboratory.

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