Transcription of Rat Liver Deoxyribonucleic Acid *in vitro* at Low Ionic Strength

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1. When RNA polymerase is in excess over DNA, the single-stranded breaks of DNA can be recognized as initiation sites for the enzyme. On the other hand, stable initiation complexes (resistant to inhibition by heparin) are the most abundant under these conditions. The formation of these complexes needs double-stranded DNA. It seems that RNA sequences rich in cytidine are preferentially synthesized; since rat liver DNA is A+T-rich, the transcription thus appears not to be random with respect to the base composition of DNA. 2. When the template is in excess over the polymerase, the single-stranded gaps of DNA are preferentially transcribed by rat liver RNA polymerase B, and native DNA regions by *Escherichia coli* RNA polymerase. 3. With a large excess of DNA over the polymerase, the enzyme activity is markedly inhibited. This inhibition is proportional to the concentration of double-stranded DNA ends, but it also depends on the presence of a contaminant of DNA, removed when DNA is banded in a CsCl gradient. This contaminant could be polyphosphates. Low concentrations of spermine completely reverse this inhibition, by enhancing the rate of RNA chain elongation. 4. Double-stranded RNA is synthesized in great abundance when RNA polymerase is in excess over native DNA. Besides a majority of symmetrical sequences, stable 'hairpins' can be found. Whereas the synthesis of symmetrical sequences is more prevalent in polymerase excess, it seems that the proportion of stable 'hairpins' in RNA is independent of the polymerase/DNA ratio.

I have found (Pays, 1976, 1977) that, at low ionic strength *in vitro*, rat liver RNA polymerase B (EC 2.7.7.6) directs the synthesis on chromatin or DNA of short RNA sequences containing a high proportion of double-stranded segments (dsRNA). A large fraction of these segments seem to be formed by intramolecular base-pairing (Pays, 1977). The synthesis of these dsRNA molecules could be related to specificity of transcription, since it is more prevalent on chromatin than on deproteinized DNA, and with homologous than with bacterial RNA polymerase. The possibility was suggested that some intramolecular complementary DNA sequences could be recognized by RNA polymerase as promoter sites. Therefore I decided to examine the interaction of RNA polymerase with purified DNA under the conditions giving rise to the synthesis of dsRNA.

Materials and Methods

DNAase I (EC 3.1.4.5) (RNAase-free) was from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; RNAase A (EC 3.1.4.22) and RNAase T1 (EC 3.1.4.8) were from Sigma, St. Louis, MO, U.S.A.; *Aspergillus* nuclease S1 (EC 3.1.4.-) was

prepared from amylase as described by Vogt (1973); *Tritirachium* proteinase K (EC 3.4.21.-) was from Merck, Darmstadt, Germany; hydroxyapatite (DNA grade) was from Bio-Rad Laboratories, Richmond, CA, U.S.A.; Soluene was from Packard, Downers Grove, IL, U.S.A.; Omnifluor was from New England Nuclear, Boston, MA, U.S.A.

RNA polymerase was extracted and purified, from either *Escherichia coli* or rat liver, as previously described (Pays & Ronsse, 1973). The different DNA samples I used were all prepared starting from rat liver DNA purified from nuclei as previously described (Pays, 1976), but with extensive RNAase A and proteinase K digestions instead of phenol extractions; this DNA preparation is referred to as 'control' DNA, or 'DNA containing gaps'. Its mean molecular weight, determined by analytical centrifugation (Studier, 1965), was 10^7 in neutral buffer, and 2.5×10^6 in denaturing conditions. This DNA contained about 5.5% single-stranded sequences (that is, sequences sensitive to nuclease S1 activity).

Denatured DNA was obtained by heating for 5 min at 100°C in 10 mM-Tris/HCl (pH 8), then quickly cooling in an ice bath just before use.

'DNA without gaps' was prepared by treating control DNA with nuclease S1 as previously described (Pays & Ronsse, 1975). Its mean mol.wt. was 2.8×10^6 in native form.

Abbreviations used: dsRNA, double-stranded RNA; RNAase, ribonuclease; DNAase, deoxyribonuclease.

'Palindromes with tails' were obtained by hydroxyapatite fractionation of sonicated DNA (mol.wt. 1.7×10^5 in denaturing conditions) as described (Pays & Ronsse, 1975), immediately after denaturation of DNA. I collected only sequences passing through hydroxyapatite in 0.5M-sodium phosphate buffer (pH 6.8) at 60°C, after having been retained on the column in 0.16M-sodium phosphate (pH 6.8) at the same temperature. This DNA preparation was 53% sensitive to nuclease S1, and thus contained 47% double-stranded sequences.

'DNA without palindromes' was the fraction of sonicated and denatured DNA passing through hydroxyapatite in 0.16M-sodium phosphate (pH 6.8) at 60°C. It contained no more than 1% of sequences resistant to nuclease S1 attack immediately after denaturation. This DNA was always re-denatured before use. A fraction of this preparation was re-natured to about 70% at a $C_0t = 10^4 \text{ M} \cdot \text{s}$ in 0.6M-NaCl/20mM-EDTA/40mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/HCl (pH 7.0), and is referred to as 'double-stranded DNA without palindromes'.

'Palindromes' were obtained by digestion of denatured DNA with nuclease S1 as described (Pays & Ronsse, 1975). This fraction represented between 4.7 and 4.9% of total DNA, and its mol.wt. was about 10^5 under denaturing conditions.

'Nicked DNA' was DNA treated at 100 µg/ml for 1 h at 37°C with 25 ng of DNAase I/ml in 0.1M-NaCl/10% glycerol/20mM-MgCl₂/50mM-Tris/HCl (pH 8). Its mol.wt. was 4.6×10^6 in native form, and 2×10^5 when denatured. A fraction of this DNA preparation was treated with nuclease S1 in low salt concentration as described by Dreyer & Hausen (1976), specifically to destroy DNA strands opposite nicks, converting these into double-stranded ends. Another fraction of this DNAase-treated DNA was treated with nuclease S1 in high salt concentration to digest the single-stranded sequences (Vogt, 1973).

The transcription assays were as described (Pays, 1976). Unless specified, they always contained the same amount of polymerase: either 1 µg of *E. coli* polymerase (80% pure, as revealed by polyacrylamide-gel electrophoresis; Burgess, 1969) or an amount of RNA polymerase B from rat liver leading to an equivalent extent of transcription on control DNA. In each case it represented 10 µl of enzyme solution. The amount of polymerase per assay was not defined as units of activity, since the specific activity of the enzymes varies according to the properties of the template and the polymerase/DNA ratio (see below). The incubation mixture (usually 0.5 ml) always contained 50mM-Tris/HCl (pH 8), 5mM-dithiothreitol, 0.1mM-ATP, 0.1mM-GTP, 16 µM-[³H]UTP and 16 µM-[³H]CTP, 5mM-(NH₄)₂SO₄ and 2mM-MnCl₂. At the end of the incubation period, RNA was precipitated in cold 10% (w/v) trichloro-

acetic acid containing 25mM-pyrophosphate, together with 50 µg of bacterial tRNA as carrier. It was then filtered on GF/C filters (Whatman, Clifton, NJ, U.S.A.) and rinsed thoroughly with trichloroacetic acid. Counting of radioactivity incorporated from [³H]UTP and [³H]CTP into RNA was performed in Omnifluor (4g/litre of toluene) containing 5% Soluene and 0.1% water, to minimize self-absorption of the radioactivity in the material precipitated on filters, as pointed out by Dynan *et al.* (1977).

dsRNA content in the transcripts was measured by incubation of RNA for 2 h at 37°C in 0.3M-NaCl/10mM-Tris/HCl (pH 8) in the presence of 100 µg of RNAase A/ml + 150 ng of RNAase T1/ml, followed by precipitation in trichloroacetic acid and counting of radioactivity of the precipitate. The control was run in the same way but without RNAase. Material resistant to RNAase in high salt was considered as double-stranded (Pays, 1976).

Results

Transcription efficiency: general characteristics

At low ionic strength [5mM-(NH₄)₂SO₄] the efficiency of transcription of rat liver DNA by either *E. coli* or rat liver (form B) RNA polymerase is about one-quarter that at the optimal salt concentration [100mM-(NH₄)₂SO₄] (E. Pays, unpublished work). As pointed out by Richardson (1970), under these conditions each RNA polymerase molecule does not seem to re-initiate easily. Indeed 70–80% of [³²P]-GTP is incorporated into RNA during the initial 15 min of incubation (results not shown); moreover, the amount of RNA synthesized does not exceed the amount of DNA template, at any of the polymerase/DNA ratios tested (Fig. 1). With rat liver RNA polymerase, a maximum of 0.4 ng of RNA is synthesized/ng of native DNA template (Fig. 1, curves 1–5), whereas the transcription is about 2.5 times more efficient on denatured DNA (Fig. 1, curves 6 and 7).

Although 0.1 mM is the optimum nucleotide concentration, and has been used for ATP and GTP, the use of only 16 µM-UTP and -CTP results in a 2-fold decrease in RNA synthesis, compared with that at the optimum 0.1 mM concentration. However, I used these conditions because they gave rise to the synthesis of a large amount of dsRNA on chromatin (Pays, 1976). Moreover, the use of a low nucleotide concentration minimizes the polymerase activity occurring without template (E. Pays, unpublished work).

All measurements were done after 2 h synthesis at 37°C, under conditions where RNA synthesis by either of the two RNA polymerases reaches a plateau for both limiting and excessive DNA concentration (Fig. 2). From kinetics experiments, it is obvious that the initial rate of synthesis is higher with the rat liver enzyme than with the bacterial one (Fig. 2).

Fig. 1. RNA synthesis by rat liver RNA polymerase B, as a function of the DNA concentration

A fixed amount of RNA polymerase B from rat liver (10 μ l; see the Materials and Methods section) was incubated during 2 h at 37°C with control DNA ($\circ$, curve 1), DNA without gaps ($\bullet$, curve 2), control DNA purified through CsCl (∇ , curve 3), DNA without gaps purified through CsCl ($\blacktriangledown$, curve 4), or denatured DNA (Δ , curve 6). In curves 5 ($\square$) and 7 ($\blacksquare$), native and denatured DNA respectively were incubated with 20 μ l of RNA polymerase. The preparation of the different DNA fractions is described in the Materials and Methods section. The amount of synthesized RNA was calculated from the incorporated radioactivity in acid-insoluble material by knowing the specific radioactivity of the precursors UTP and CTP, and assuming that each of the four nucleotides is represented in equal proportion in RNA. The Figure is divided into three parts (A, B and C), as described in the text; these parts correspond to parts A, B and C of the Results section.

Fig. 2. Kinetics of RNA synthesis by either rat liver RNA polymerase B or *E. coli* RNA polymerase on native DNA. RNA was synthesized on either 10 ng ($\bullet$, $\circ$) or 500 ng ($\blacksquare$, $\square$) of native DNA by either 10 μ l of rat liver RNA polymerase B ($\bullet$, $\blacksquare$) or 10 μ l of *E. coli* RNA polymerase ($\circ$, $\square$). Conditions for synthesis were as described in the Materials and Methods section. Results are expressed as percentage transcription as a function of the incubation time, taking as 100% the RNA synthesis measured after 120 min incubation. 100% synthesis represented 3.3, 18.5, 2.8 and 48 ng of RNA for $\bullet$, $\blacksquare$, $\circ$ and $\square$ respectively (the estimations were done as indicated for Fig. 1).

The size of the RNA sequences is heterogeneous, from 100 to 300 nucleotides synthesized by rat liver polymerase (Figs. 3 and 4), and from 100 to 2000 nucleotides synthesized by *E. coli* polymerase on native DNA (Fig. 3). This size distribution is about the same at different polymerase/DNA ratios (results not shown), and is unchanged if RNA synthesis is performed in the presence of 1 mg of heparin/ml, a powerful inhibitor of ribonuclease activity (Palmiter, 1973) (Fig. 4). On denatured DNA, the size of the RNA sequences is larger, accounting probably to some extent for the increased nucleotide incorporation (Fig. 4).

Transcription efficiency as a function of polymerase/DNA ratio

With a fixed amount of RNA polymerase, an increase of DNA template leads to an increase of nucleotide incorporation in three stages, firstly strictly proportional to the DNA concentration when the enzyme is in excess (Fig. 1, part A), secondly less proportional (Fig. 1, part B), and finally by an inhibition of synthesis when DNA is in large excess

Fig. 3. Size distribution of the RNA sequences, as revealed by electrophoresis on 2.2% polyacrylamide gels. RNA synthesized on native DNA by either rat liver RNA polymerase B (●) or *E. coli* polymerase (○) was electrophoresed for 4 h at 6 mA/tube in 2.2% polyacrylamide/0.5% agarose gels as described by Loening (1967). Gel slices (about 2 mm long) were counted for radioactivity in 5% Soluene in toluene/Omnifluor. Arrows indicate the position of 28S, 23S, 18S, 16S and 4S RNA respectively, from left to right. In a separate experiment *E. coli* ^{14}C -labelled rRNA was added during incubation of DNA with RNA polymerase, then purified and electrophoresed together with RNA synthesized on DNA. No bacterial RNA degradation was detectable, indicating little or no RNAase activity in the incubation mixture.

Fig. 4. Size distribution of the RNA sequences, as revealed by electrophoresis on 10% polyacrylamide gels. RNA was synthesized by rat liver RNA polymerase B on denatured (○) or native DNA (■, with 1 mg of heparin/ml after 15 min preincubation without UTP, CTP and heparin; ●, without heparin), and electrophoresed for 4 h at 6 mA/tube in 10 cm long gels as described by Loening (1967). Arrows indicate the position of bacterial 5S and 4S RNA respectively, from left to right. The double arrow denotes the position of the Bromophenol Blue marker.

over the enzyme (Fig. 1, part C). These three stages are examined in the three sections below.

Part A: excess of RNA polymerase over DNA. A1: transcription from nicks. When RNA polymerase is in excess over DNA, the introduction of breaks in DNA by either mild DNAase I treatment or sonication enhances its template activity for RNA polymerase from *E. coli* (Fig. 5, curve 5) and, to a lesser extent, for RNA polymerase B from rat liver (Fig. 6, curves 2 and 3). When single-strand breaks are converted into double-strand ends by nuclease S1 treatment in low salt concentration (Germond *et al.*, 1974), this stimulation of transcription is lost, and the incorporation becomes slightly less than on unsheared DNA (Fig. 6, curves 4 and 5). This suggests, as Dreyer & Hausen (1976) have pointed out, that single-strand breaks (and not double-strand ends) can be recognized by RNA polymerase under these conditions. The stimulation of transcription with polymerase excess is only partially lost with DNAase-treated DNA digested with nuclease S1 in high salt concentration, under conditions for hydrolysis of the single-stranded sequences (Vogt, 1973) (Fig. 6, curve 6). Nicks are thus preferred over gaps under these conditions.

A2: formation of stable initiation complexes. Although this unspecific initiation on single-strand breaks is clearly seen only with an excess of RNA polymerase over DNA, I also found a higher percentage of stable initiation complexes of RNA polymerase with DNA under these conditions (Fig. 7). My criterion for stable initiation complexes is the resistance to dissociation by heparin (1 mg/ml) after preincubation of RNA polymerase with DNA at 37°C in the complete incubation medium without UTP and CTP. The formation of such initiation complexes has been reported to occur in regions containing active promoters in viral DNA (Schäfer *et al.*, 1973; Jones *et al.*, 1977). The stable initiation complexes resist a 5 min incubation at 37°C in the presence of heparin, before elongation can take place by addition of UTP and CTP. The transcription resistant to heparin after preincubation at 0°C (maximum 2.5%) is subtracted from that obtained at 37°C. The RNA molecules synthesized under these conditions are of the same length as those synthesized without heparin (Fig. 4), which suggests that heparin is without effect on RNA chain elongation on pure DNA. The percentage of radioactivity incorporated into RNA in the presence of heparin, compared with

Fig. 5. RNA synthesis by *E. coli* RNA polymerase, as a function of the DNA concentration. Conditions were the same as for Fig. 1, but with *E. coli* RNA polymerase and control DNA (○, curve 1), DNA without gaps (●, curve 2), DNA purified through CsCl (▽, curve 3), denatured DNA (△, curve 4) and sonicated DNA (■, curve 5).

Fig. 6. RNA synthesis by rat liver RNA polymerase B, as a function of the DNA concentration. Conditions were the same as for Fig. 1, but with control DNA (○, curve 1), sonicated DNA (■, curve 2), nicked DNA (●, curve 3), sonicated DNA treated with nuclease S1 in low salt (□, curve 4), nicked DNA treated with nuclease S1 in low salt (▽, curve 5), nicked DNA treated with nuclease S1 in high salt (▼, curve 6) and denatured DNA (△, curve 7).

the total without heparin, is thus a direct estimate of the percentage of stable initiation complexes. This value reaches about 33% for native DNA when RNA polymerase is in excess, and falls to about 22% when RNA polymerase becomes limiting (Fig. 7). On denatured DNA, the corresponding estimates are 27 and 9% respectively, whereas on denatured DNA from which most (80%) palindromic sequences were removed by hydroxyapatite fractionation they become 14 and 9%. Since the formation of stable initiation complexes is decreased by removal of palindromic sequences, it seems that palindromes are required to form stable complexes with RNA polymerase on denatured DNA. However, DNA devoid of most palindromic sequences, but renatured at about 70% (at C_0t $10^4 M \cdot s$ in 0.6M-NaCl) has a higher percentage of initiation complexes than does native DNA (47–27%, depending on the polymerase/DNA ratio). The formation of initiation complexes thus seems to require double-stranded DNA, but not specifically palindromic sequences. The formation of stable initiation complexes also seems not to be dependent on the amount of nicks or ends in DNA,

Fig. 7. Formation of stable initiation complexes as a function of the DNA concentration

The formation of stable initiation complexes of rat liver polymerase (10 μ l; see the Materials and Methods section) with control DNA (●), sonicated DNA (△), denatured DNA (○), denatured DNA without most palindromes (□) and double-stranded DNA without most palindromes (■) is expressed as percentage resistance to inhibition by heparin as a function of the DNA concentration. After a preincubation period of 15 min at 37°C without UTP and CTP, DNA was incubated with RNA polymerase for 5 min at 37°C in the presence of 1 mg of heparin/ml, then for 2 h with [3H]UTP and [3H]CTP at the same temperature. The stable initiation complexes thus resist a 5 min incubation in the presence of heparin, before elongation can take place. In each case the percentage of resistance to heparin obtained after preincubation at 0°C has been subtracted; it represented no more than 2.5% of incorporation without heparin.

since the percentage resistance to heparin inhibition is about the same with either control or sheared DNA (Fig. 7).

A3: preferential incorporation of cytidine over uridine. Another observation is made under conditions of polymerase excess: the transcription appears not to be random with respect to the base composition of the nucleic acid sequences. Indeed the cytidine/uridine ratio in RNA synthesized on native DNA is significantly higher than the cytidine/thymidine ratio in rat DNA. Although cytidine comprises 42.55% of total pyrimidines in rat DNA (Steele, 1968), it represents 55.5–44.8% of total pyrimidines in RNA synthesized on native DNA, depending on the polymerase/DNA ratio and the time of incubation (Table 1 and Fig. 8). The preferential incorporation of cytidine is the highest at high polymerase/DNA ratio and after short times of incubation. On the other hand, transcription on denatured DNA does not exhibit such nucleotide selection, since the ratio of incorporation of cytidine to total pyrimidines in RNA transcribed on denatured DNA is the same as, or even slightly lower than, in DNA itself (Table 1 and Fig. 8). The preferential incorporation on G·C base-pairs of native DNA was equally observed with different DNA fractions separated on a CsCl gradient according to their base composition (results not shown).

Another type of experiment also suggests some selectivity in the choice of base-sequence transcription on native DNA: there is a small but reproducible difference in temperature between that needed for denaturation of the template leading to a maximal stimulation of transcription and that measured for overall 'melting' of DNA (Fig. 9). With either a high polymerase/DNA ratio with rat liver (Fig. 9a) or *E. coli* enzyme (Fig. 9b), or a low ratio with rat liver polymerase (Fig. 9c), the temperature needed to reach the mid-value of maximal stimulation of transcription is 2.5°C lower than the 'melting' temperature of the DNA. The same difference of temperature was also obtained when transcription was conducted in the presence of all the four nucleotides in high concentration (results not shown), under

Table 1. Ratio of incorporation of cytidine to total pyrimidines in RNA synthesized at different RNA polymerase/DNA ratios

Values are expressed as CMP incorporated as a percentage of (CMP+UMP) incorporation in 10 min at 37°C with rat liver RNA polymerase.

RNA polymerase (μ l/ml) ...	10	50	100
DNA (ng/ml)			
10	50.5	54.0	55.5
50	46.0	48.4	49.1
100	45.2	47.0	48.1
200	44.8	45.4	46.3
Denatured DNA (50ng/ml)	42.5	43.1	43.3

Fig. 8. Ratio of CMP incorporation to (UMP+CMP) incorporation into RNA, as a function of the incubation period with RNA polymerase from either (a) rat liver or (b) *E. coli*

The ratio of incorporation of cytidine to total pyrimidines is expressed as a function of the incubation period under different conditions: excess of RNA polymerase over native DNA (20 ng of DNA/ml) (○); slight excess of native DNA over polymerase (100 ng of DNA/ml) (□); large excess of native DNA over polymerase (1 μg of DNA/ml) (△); each of the three polymerase/DNA ratios cited, but with denatured DNA (●).

Fig. 9. Stimulation of DNA transcription after thermal denaturation of the template

The transcription of DNA (20 ng/ml) was performed immediately after heating of the template for 5 min in 50 mM-Tris/HCl (pH 8). The conditions of transcription varied as follows: (a) standard incubation mixture (containing 2 mM-MnCl₂) with rat liver polymerase in the presence of 0.1 mM-ATP and -GTP, 16 μM-[³H]UTP and -[³H]CTP; (b) as (a), but with *E. coli* polymerase; (c) as (a), except that the DNA concentration was 2 μg/ml; (d) as (a), but with 6 mM-MgCl₂ instead of 2 mM-MnCl₂. The RNA synthesis is expressed as percentage of maximal incorporation (○); 100% incorporation represents 14517, 23940, 45586 and 1830 c.p.m., for (a), (b), (c) and (d) respectively. In (a) is shown the thermal denaturation profile of DNA, under the conditions for the transcription assays, as revealed by hyperchromicity at 260 nm, expressed as percentage of maximal hyperchromicity (●). The first arrow, at the left, indicates the temperature at which half-maximal stimulation of transcription is observed. The second arrow indicates the 'melting' temperature of DNA.

conditions known to favour transcription of native rather than denatured DNA (Dezélée & Sentenac, 1973; Dynan *et al.*, 1977). Moreover, the same difference of temperature was again obtained when 6 mM-MgCl₂ was used instead of 2 mM-MnCl₂ (Fig. 9d), under conditions favouring stabilization of the DNA double helix (Hossenlopp *et al.*, 1974; Mandel & Chambon, 1974). The transcription thus seems to be particularly stimulated by the denaturation of DNA sequences 'melting' at about 2.5°C below the mean 'melting' temperature of DNA. Since these sequences must be A+T-rich, it can be concluded that A+T-rich regions have to be denatured to be transcribed, and thus are probably less efficiently transcribed than G+C-rich ones when rat liver DNA is in native form.

Part B: excess of DNA over RNA polymerase. Transcription of single-stranded gaps. When RNA polymerase becomes limiting in the transcription assay with DNA, the single-stranded sequences of DNA are better recognized by DNA polymerase B from rat liver, whereas *E. coli* polymerase prefers the double-stranded sequences. As Fig. 1 (part B) shows, treatment of DNA with nuclease S1 to destroy single-stranded sequences selectively results in a 2-fold restriction of template activity for rat polymerase B (Fig. 1, curves 1, 2, 3 and 4). This effect appears likely

to be due to hydrolysis of the single-stranded sequences. It means that the single-stranded sequences are particularly well transcribed by rat polymerase B under these conditions. With *E. coli* polymerase, DNA treated with nuclease S1 is equally well transcribed as untreated DNA under the same conditions (Fig. 5, curves 1, 2 and 3), indicating no preferential transcription of the single-stranded sequences; moreover, in this part of the curve, the polymerase of *E. coli* transcribes native DNA more efficiently than denatured DNA (Fig. 5, curves 1 and 4).

Part C: large excess of DNA over RNA polymerase. C1: inhibition by polyphosphates. At high DNA/polymerase ratios, transcription is markedly inhibited, for both rat liver (Fig. 1, curves 1 and 2) and *E. coli* (Fig. 5, curves 1 and 2) RNA polymerases. This inhibition is inversely proportional to the size of

Fig. 10. Transcription of DNA at high concentration of template, as a function of the size of the DNA sequences. DNA was fractionated on Sepharose 4B (Pharmacia, Uppsala, Sweden) as described by Flint *et al.* (1974), then transcribed by 10 μ l of RNA polymerase B from rat liver (see the Materials and Methods section). Fractions of mol.wt. 10^7 native (2×10^6 denatured) (○), 7.5×10^6 native (1.4×10^6 denatured) (●) and 6×10^6 native (1.1×10^6 denatured) (□) were assayed. Another assay was conducted on a DNA fraction of mol.wt. 10^6 , arising from a DNA preparation banded in CsCl before fractionation (△). The amount of RNA synthesized is expressed as percentage of that found with 1 μ g of DNA template.

the DNA sequences (Fig. 10). This strongly suggests that the inhibition is related to the concentration of DNA ends. However, it is obvious that DNA purified by banding in a CsCl gradient remains well transcribed at high concentration, either by rat liver (Fig. 1, curves 3 and 4) or *E. coli* (Fig. 5, curve 3) RNA polymerase, even if the DNA is of a low molecular weight (Fig. 10). The inhibition of transcription at high DNA concentration appears thus to be due to a contaminant removed by centrifugation in CsCl. This contaminant is recovered in the bottom fractions of the centrifuged solution, since, after dialysis, the latter fractions are able to inhibit DNA transcription (results not shown). As Ponta *et al.* (1974) have pointed out, I also observed that the inhibitory factor distribution parallels the distribution of polyphosphates in CsCl gradients (results not shown). Polyphosphates, whatever their chain length (I have used polyphosphates averaging from 5 to 200 residues, purchased from Sigma), inhibit transcription of DNA *in vitro*. I obtained half-maximal inhibition in about 10 μ M-phosphate, and a maximal inhibition above 1 mM-phosphate.

Fig. 11. Transcription of DNA as a function of spermine concentration.

RNA polymerase B from rat liver (10 μ l; see the Materials and Methods section) was incubated during 2 h at 37°C with different amounts of DNA and different concentrations of spermine [300 μ M (△), 30 μ M (■), 3 μ M (●), or without spermine (○)]. The amount of synthesized RNA was calculated as for Fig. 1.

Table 2. Effect of spermine on initiation and elongation. Spermine (0.3 mM) was added (+) or not (○) either during preincubation of RNA polymerase with DNA in the absence of UTP and CTP or during incubation in the presence of UTP, CTP and heparin (50 μ g/ml). The preincubation and incubation times were respectively 15 min and 2 h at 37°C. These conditions are used for synthesis of RNA initiated in stable initiation complexes, as described for Fig. 7. The DNA concentration was 2 μ g/ml, with RNA polymerase from *E. coli*. The blank value without spermine or heparin was 2009 c.p.m. Background incorporation (about 50 c.p.m.) has been subtracted in each case.

Addition of spermine during		UMP incorporation (c.p.m.)
Preincubation (without heparin)	Incubation (with heparin)	
○	○	550
○	+	3577
+	○	635

C2: stimulation by spermine. At high DNA/polymerase ratios, the inhibition of transcription can be reversed by the addition of spermine (Fig. 11). Maximal stimulation is obtained in 300 μ M-spermine; above that concentration inhibition again occurs (result not shown). The effect of spermine is at the level of RNA elongation, as shown in Table 2. Indeed, stimulation of transcription is obtained when spermine is added to the incubation mixture of RNA polymerase with DNA under conditions leading only to elongation of RNA, that is in the presence of

Fig. 12. Stabilization by salt of dsRNA sequences in RNA synthesized on 10 µg of DNA by either *E. coli* (○) or rat liver (●) RNA polymerase

Resistance of transcripts to RNAase A (100 µg/ml) + RNAase T1 (150 ng/ml) was assayed as a function of NaCl concentration in 10 mM-Tris/HCl (pH 8). The incubation with RNAase was for 2 h at 37°C; RNA sequences precipitated in 10% trichloroacetic acid at the end of the incubation period were considered as double-stranded (Pays, 1976). The resistance of dsRNA to RNAase is expressed as a percentage of that found in 0.3 M-NaCl. At this salt concentration dsRNA represented 7.1% of total RNA transcribed by rat liver polymerase, and 4.1% of RNA transcribed by *E. coli* polymerase. The arrow indicates the salt concentration needed to reach half-maximal stabilization of dsRNA.

heparin. If spermine is added during the formation of stable initiation complexes, no stimulation is found, suggesting that spermine inhibits initiation.

Synthesis of double-stranded sequences

As previously mentioned (Pays, 1976), both *E. coli* and rat liver RNA polymerases synthesize dsRNA on rat liver DNA at low ionic strength. Complete stabilization of the dsRNA (that is, complete resistance to RNAase attack) is attained at a relatively high salt concentration (375–450 mM-NaCl; see Fig. 12), compared with that needed for stabilization of dsRNA synthesized on chromatin by rat liver polymerase (320–340 mM-NaCl; Pays, 1977). This suggests that dsRNA synthesized on DNA could be more mispaired than dsRNA synthesized on chromatin by the homologous RNA polymerase (Pays, 1977). The salt concentration needed for stabilization of dsRNA is the same for transcripts by either *E. coli* or rat liver RNA polymerase (Fig. 12). This contrasts with what has been observed for dsRNA synthesized on chromatin, where the stabilization of the transcripts synthesized by the rat enzyme was attained at

Fig. 13. Denaturation curves of dsRNA sequences included in RNA transcripts

The resistance to RNAase was measured for RNA synthesized by rat liver polymerase on 25 ng of native (a) or denatured (c) DNA, and for RNA synthesized by the same enzyme on 5 µg of native (b) or denatured (d) DNA. In each case incubation of RNA was performed for 2 h at 37°C in 40% (v/v) formamide/0.3 M-NaCl/50 mM-Tris/HCl (pH 8), after 5 min preincubation at the temperature indicated. RNAase A (100 µg/ml) + RNAase T1 (150 ng/ml) were added during (●) or after (○) the preincubation period. Resistance to RNAase is expressed as a percentage of that found where preincubation is performed at 37°C; 100% resistance corresponds to 40% of total RNA and 1092 c.p.m. for (a), 9% and 2270 c.p.m. for (b), 16.5% and 1580 c.p.m. for (c), 4.5% and 4222 c.p.m. for (d).

lower NaCl concentration than for transcripts synthesized by the *E. coli* polymerase (Pays, 1977).

As observed before for dsRNA synthesized on chromatin (Pays, 1976), the thermal denaturation curve of dsRNA synthesized on DNA is different whether the dsRNA sequences are freed or not from the single-stranded contiguous sequences (Fig. 13). Indeed the denaturation of dsRNA, measured by resistance to RNAase in high salt concentration, is only complete when RNAase is present during heating (Fig. 13). This strongly suggests that a single-stranded link, which can be hydrolysed by RNAase in high salt concentration, permits the very fast renaturation of a portion of dsRNA on cooling. If RNAase is added only after heating, presumably it can no longer attack a fraction of dsRNA rapidly renatured; on the contrary, this fraction of dsRNA cannot renature if

Table 3. *Percentage of dsRNA in transcripts as a function of the DNA concentration*

Values are expressed as percentage of dsRNA in total transcripts. In each case the saturation of DNA by RNA polymerase is obtained below 50 ng of DNA/ml (see the transition between part A and part B of the incorporation curves of Figs. 1 and 2); 10 μ l of polymerase solution was used in each assay (see the Materials and Methods section).

DNA (ng/ml) ...	10	100	500	2000	10000
RNA polymerase from:					
Rat liver	40.5	37.1	35.1	16.7	9.8
<i>E. coli</i>	18.9	17.9	21.0	13.3	8.4

Table 4. *Percentage of dsRNA in transcripts as a function of the nature of the DNA template*

Values are expressed as percentage of dsRNA in total transcripts. In each case the concentration of DNA in the transcription assay was 1 μ g/ml, with 10 μ l of rat liver RNA polymerase per assay, as in Table 3. Conditions of DNA preparation and characterization, as well as determination of dsRNA content in the transcripts, were as described in the Materials and Methods section.

DNA template	dsRNA (%)
Native (double-stranded with gaps)	19.7
Native, treated with nuclease S1 (double-stranded without gaps)	17.7
Denatured (single-stranded with palindromes)	9.7
Denatured, eluted as single-stranded on hydroxyapatite (single-stranded without most palindromes)	10.1
Denatured, eluted as double-stranded on hydroxyapatite (palindromes with tails)	16.2
Denatured, treated with nuclease S1 (palindromes)	20.3
Denatured, eluted as single-stranded on hydroxyapatite, then renatured (double-stranded without most palindromes)	19.9

RNAase is present and active during the denaturation. The fraction of dsRNA resistant to RNAase after having been heated under conditions for complete denaturation can thus be considered as a minimal estimate of intramolecularly base-paired dsRNA. In the four cases I have examined (RNA synthesized at two polymerase/DNA ratios, on either native or denatured DNA), this fraction of dsRNA, presumably in 'hairpin' loops, is between 2 and 4% of total transcripts (Figs. 13a–13d). Since the percentage of total dsRNA is 40% and 9% respectively for RNA transcribed with a large excess or a limiting amount of RNA polymerase on native DNA (Figs. 13a and 13b; Table 3), and 16% and 4% for RNA synthesized under the same conditions but on denatured DNA (Figs. 13c and 13d), it could be that a large fraction of dsRNA is not in 'hairpin' loops, especially at high

Fig. 14. *Fractionation on Sephadex G-100 of RNA synthesized on 10 μ g of DNA by *E. coli* RNA polymerase* RNA, in 0.5 ml of 0.3M-NaCl/10mM-EDTA/10mM-Tris/HCl (pH8), was fractionated by elution on a column (75 cm $\times$ 1 cm) of Sephadex G-100 at a rate of 15 ml/h; 1.5 ml fractions were collected. The radioactivity of each fraction was determined for 50 μ l samples in 33% (v/v) Triton X-100 in Omnifluor (●). The positions of *E. coli* 5S- and 4S-RNA markers, eluted under the same conditions, are shown. The percentage of dsRNA sequences was determined in some fractions (○).

polymerase/DNA ratio. In fact, several observations can be interpreted in the sense that the majority of dsRNA arises from symmetrical transcription of the two opposite DNA strands, and that this symmetrical transcription is more prevalent when RNA polymerase is in excess over DNA: (1) the proportion of dsRNA in transcripts is higher at high polymerase/DNA ratios, with rat liver as with *E. coli* polymerase (Table 3), whereas the synthesis of 'hairpin' loops is independent of the polymerase/DNA ratio (Fig. 13); (2) the proportion of dsRNA is lower for transcripts of denatured DNA (Fig. 13 and Table 4), and is not directly correlated with the presence of palindromes in DNA (Table 4); (3) the proportion of dsRNA is higher in the larger transcripts (Fig. 14).

Discussion

The present observations on the transcription of rat liver DNA at low ionic strength suggest that only some sequences can be recognized by the RNA polymerase.

(1) Sequences on which initiation of transcription proceeds are double-stranded

Many authors (Gniazdowski *et al.*, 1970; Dezélee & Sentenac, 1973; Dezélee *et al.*, 1974; Hossenlopp *et al.*, 1974) have claimed that eukaryotic enzymes are unable to 'read' intact double-stranded DNA without an unpaired region. However, my results seem to indicate that the formation of stable initiation complexes requires a double-stranded sequence, since the amount of these complexes is decreased on denatured DNA, especially when palindromic sequences are removed. In addition, the formation of stable initiation complexes does not depend on the amount of nicks or ends in DNA. The transcription is dependent on the presence of single-stranded regions in DNA only when the polymerase is limiting. Indeed, with excess DNA over the polymerase, the transcription is strongly stimulated when DNA contains single-stranded gaps. This result confirms previous observations by Tsai *et al.* (1975). On the contrary, with excess polymerase over DNA, the transcription efficiency is the same for either DNA containing gaps, or DNA completely double-stranded after treatment with nuclease S1. This is also true for purified palindromes (results not shown). It can be deduced that stable initiation complexes can be formed on DNA sequences having low affinity for polymerase, since this process requires excess of enzyme. A plateau is, however, attained when the RNA polymerase concentration is increased, indicating that there is a limited number of sequences able to form stable initiation complexes with RNA polymerase. Similar observations have been made by Meilhac & Chambon (1973) with RNA polymerase B from calf thymus, and by Long & Crippa (1976), with RNA polymerase C from *Xenopus laevis*.

The fact that native unsheared double-stranded DNA is often considered as a poor template for transcription *in vitro* by eukaryotic RNA polymerases (Dezélee & Sentenac, 1973; Dezélee *et al.*, 1974) could be due, at least partially, to a strong inhibition by a contaminant of DNA. My results on this point agree entirely with those obtained by Ponta *et al.* (1974). The contaminant could be either polyphosphates or residual RNA fragments, since both have been reported to inhibit transcription (Sasaki *et al.*, 1974) and can be separated from DNA by centrifugation in CsCl. It is, however, very unlikely that RNA remains in the DNA preparation, since extensive RNAase treatment in low-salt buffer was carried out during the purification of DNA. Therefore, I suggest, as

did Ponta *et al.* (1974), that this contaminant could be polyphosphates. It seems that the usual DNA concentrations for transcription assays (above 1 µg/ml) are sufficient for inhibition by the contaminant to occur. Double-stranded DNA ends seem to play the role, or potentiate the effect, of this contaminant, since shearing of DNA, by either DNAase treatment or sonication, leads to a greater inhibition of transcription at high DNA concentration (Flint *et al.*, 1974). Alternatively, shearing of DNA could lead to release of phosphates, known as inhibitors of the formation of phosphodiester bonds (Maitra & Hurwitz, 1967). The inhibition by the contaminant can be reversed by the addition of spermine. The simplest interpretation would seem to be that spermine binds polyphosphates; however, spermine also stimulates transcription of DNA devoid of polyphosphates (after centrifugation in CsCl) (results not shown). In fact, spermine seems to stimulate mainly RNA elongation, as shown by experiments where spermine is added after the initiation step. Mandel & Chambon (1974) also concluded that elongation of RNA is stimulated by spermine at low ionic strength. As for inhibition by the contaminant, stimulation by spermine only occurs with limiting polymerase amounts. I suggest that spermine acts at the level of single-stranded DNA regions, since its effect is obtained only under conditions where single-stranded sequences are particularly well transcribed.

Whereas the formation of stable initiation complexes is more prevalent at a high polymerase/DNA ratio, other processes occur under the same conditions: the initiation of transcription on single-stranded ends, and the symmetrical transcription of the two opposite DNA strands. Initiation on DNA breaks is particularly evident with *E. coli* RNA polymerase, but it is also noted with the rat liver enzyme. Since the formation of stable initiation complexes is independent of DNA shearing, initiation complexes on DNA ends or nicks are probably unstable, as pointed out by Meilhac & Chambon (1973). Symmetrical transcription is supposed to occur at high polymerase/DNA ratio, since under these conditions a high proportion of the RNA transcripts is double-stranded. In contrast with what has been observed in chromatin transcripts (Pays, 1977), the majority of these dsRNA sequences can be denatured by heating, suggesting that dsRNA is largely intermolecularly base-paired, and thus probably formed principally by symmetrical transcription. However, a small fraction of RNA (2–4% of total transcripts) seems to renature rapidly after denaturation, as expected for intramolecularly base-paired RNA, in 'hairpin loop'-like sequences. Whereas the synthesis of dsRNA in 'hairpin loops' is independent of the polymerase/DNA ratio, symmetrical transcription seems to be more important when RNA polymerase is in excess over DNA.

It could be argued that the dsRNA sequences that I find in the transcription products are formed by copying of endogenous RNA, as noted by Zasloff & Felsenfeld (1977) and Giesecke *et al.* (1977). In this study, however, the DNA template has been freed of RNA by extensive RNAase treatment (100 µg/ml, 2 h at 37°C in low salt concentration) during its purification procedure. Moreover, the percentage of dsRNA in the transcripts is always about the same during the synthesis (measurements made from 5 to 120 min incubation; results not shown), indicating that the first RNA molecules synthesized do not seem to serve as template for synthesis of dsRNA.

(2) Sequences on which initiation of transcription occurs are selected

I found that the proportion of cytidine is far higher in RNA synthesized at high polymerase/DNA ratio and short incubation periods than in the overall base composition of DNA. This means that guanine-rich regions are selectively recognized by RNA polymerase in DNA. No such preferential incorporation of cytidine is found on denatured DNA, suggesting that the specific recognition of guanine-rich sequences needs double-strandedness of DNA, as it was the case for the formation of stable initiation complexes.

The transcription of DNA thus appears not to be random with respect to the base composition of the transcription products. Alonso *et al.* (1976), using RNA/DNA hybridization methods, have shown that mouse DNA is not randomly transcribed by *E. coli* RNA polymerase. The same observation was made for calf thymus DNA by Atikkan & Furth (1977). Moreover, Meilhac & Chambon (1973) and Smuckler & Tata (1972) have observed that RNA polymerases A and B have different initiation sites on pure DNA. Finally, Beebee & Butterworth (1974) have pointed out that RNA polymerase B never synthesizes rRNA sequences on DNA. All these results also suggest that there is a high selectivity in the choice of the DNA sequences to be transcribed, by either *E. coli* or eukaryotic RNA polymerase.

(3) Summary of the observations; conditions for 'faithful' initiation in vitro

The evident 'non-random' nature of transcription with an excess of polymerase over DNA, as well as the greater (but limited) amount of stable initiation complexes under these conditions, could be taken as indications of good promoter recognition by the RNA polymerase when the enzyme is in excess. However, under these conditions symmetrical transcription is possible, as well as initiation on single-stranded DNA ends. This latter artifact is, however, not as important as in DNA transcription by RNA polymerase A

(Butterworth *et al.*, 1973; Van Keulen & Retel, 1977). This result is expected if we assume that RNA polymerase A has only few promoters to recognize in the DNA, and is thus highly affected by the introduction of new initiation points, whereas this is not true for RNA polymerase B. The conditions for proper initiation could thus not be the same in the two cases. Therefore a low enzyme/DNA ratio should not be considered *a priori* as the best condition for specific initiation by RNA polymerase B, on the basis of the results published for RNA polymerase A (Van Keulen & Retel, 1977). Moreover, with a low polymerase/DNA ratio the single-stranded gaps of DNA are preferentially transcribed. Although the exact significance of these gaps is unknown, it is worth mentioning that the high affinity of RNA polymerase B for single-stranded DNA sequences could prevent the specific binding of the polymerase to the DNA promoters when DNA is in excess over the enzyme. As already pointed out (Meilhac & Chambon, 1973; Schäfer *et al.*, 1973) the initiation complexes formed at DNA nicks or breaks are unstable; proper initiation by the RNA polymerase is thus possible even on sheared DNA, provided that 'DNA-like' competitors (i.e. heparin) are added in the incubation mixture after preincubation of the polymerase with DNA.

My experiments suggest that some palindromic sequences can be transcribed: indeed I have observed the synthesis of double-stranded RNA sequences with properties expected for intramolecularly base-paired RNA. This particular synthesis seems to be independent of the polymerase/DNA ratio.

Some palindromes could be implicated in the formation of stable initiation complexes, since their removal decreases the amount of stable initiation complexes on denatured DNA. These results are compatible with the idea of an initiation near or in intramolecular complementary sequences, as I suggested for rat liver chromatin (Pays, 1976), or as it can be inferred from the results of Shen & Hearst (1977) for SV40 DNA (see also Soeda *et al.*, 1977, for other DNA species).

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