Characterization of Double-Stranded Ribonucleic Acid Sequences Present in the Initial Transcription Products of Rat Liver Chromatin

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(Received 30 September 1976)

At low ionic strength and with a low exogenous RNA polymerase/DNA ratio, rat liver chromatin directs the synthesis *in vitro* of RNA sequences rich in double-stranded segments. All the transcripts contain at least one double-stranded sequence. Most of the double-stranded segments are formed by intramolecular base-pairing of inverted complementary sequences separated by a single-stranded loop. They are heterogeneous in size, 35–45% of them being more than 80 nucleotides long. They contain 61–64% G+C, whether synthesized by rat liver RNA polymerase (form B) or *Escherichia coli* RNA polymerase. The largest double-stranded sequences are found in the largest transcripts, and are the most thermostable. The fidelity of base-matching is better in double-stranded transcripts synthesized on rat liver chromatin by homologous polymerase than in those synthesized on it by a bacterial polymerase, or in those synthesized by either of the two polymerases on pure DNA.

The transcription of chromatin *in vitro* by exogenous bacterial or eukaryotic RNA polymerase (EC 2.7.7.6) appears to mimic chromatin activity *in vivo* (Paul & Gilmour, 1968; Axel *et al.*, 1973; Astrin, 1973; Gilmour & Paul, 1973; Shih *et al.*, 1973). This system provides an opportunity for studying the initial products of transcription in a eukaryotic organism. I have found (Pays, 1976) that the chromatin transcripts are particularly rich in dsRNA* sequences. Essentially two pieces of evidence were presented: (1) some sequences are resistant to RNAase A (EC 3.1.4.22) and RNAase T1 (EC 3.1.4.8) in high-salt buffer, even after extensive DNAase (EC 3.1.4.5) treatment; (2) RNAase III (EC 3.1.4.24; specific for dsRNA), but not RNAase H (EC 3.1.4.34; specific for RNA–DNA hybrids), can digest all of these resistant sequences. These results, however, did not allow us to distinguish whether the double-stranded sequences arise from symmetrical transcription of both strands of DNA, or from transcription of inverted complementary sequences (palindromes) in one DNA strand.

The percentage resistance to RNAase was higher in chromatin transcripts than in RNA molecules synthesized on pure DNA; it is higher with homologous rat liver RNA polymerase B than with *Escherichia coli* RNA polymerase (Pays, 1976). These observations suggest that some specificity could be linked to the presence of dsRNA in the transcription products.

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

Finally, like others (Jelinek & Darnell, 1972; Ryskov *et al.*, 1972; Brinker *et al.*, 1973), I have found the same type of dsRNA sequences in heterogeneous nuclear RNA from rat liver.

I present here further characterization of these dsRNA sequences. It is suggested that most of them are formed by intramolecular base-pairing, rather than by hybridization of symmetrical sequences from different molecules; moreover, the complementary sequences seem to be separated by a single-stranded piece. The stability of dsRNA molecules of different sizes or origins is also detailed, with special emphasis on the comparison between transcripts by homologous or bacterial RNA polymerase.

Methods

Purification and synthesis

Purification of rat liver chromatin and its transcription in low-salt buffer [5 mM-(NH₄)₂SO₄] were performed as described by Pays (1976). Chromatin was not sheared and was not stored longer than 24 h at 4°C. RNA polymerase B from rat liver, and RNA polymerase from *E. coli* (holoenzyme), were extracted and purified as described (Pays & Ronsse, 1973). *E. coli* RNA polymerase was about 80% pure, as revealed by slab-gel electrophoresis in denaturing conditions (Burgess, 1969). Rat liver RNA polymerase B was sometimes found associated with residual DNA fragments. In this case the enzyme was treated with DNAase I (RNAase-free, from

Worthington Biochemical Corporation, Freehold, NJ, U.S.A.; 20 µg/ml for 45 min at 37°C) and rechromatographed on DEAE-Sephadex as described previously (Pays & Ronsse, 1973) to inhibit enzymic activity occurring in the absence of exogenous template. The enzyme/DNA ratio was about 1:10 (w/w) in the reaction mixture, which contained 10 µg of DNA/0.5 ml. The RNA remained fully trichloroacetic acid-precipitable for at least 8 h in the incubation medium with chromatin at 37°C; no RNAase activity was thus detectable within this period.

RNA was isolated from the synthesizing system *in vitro* by extensive proteinase digestions and phenol/chloroform extractions as described previously (Pays, 1976). It could be completely hydrolysed in 0.3 M-KOH in 3 h at 37°C. Centrifugation in Cs₂SO₄ gradients was performed as described previously (Pays, 1976) to ensure the absence of residual DNA after the drastic DNAase treatment included in the purification procedure. RNA was never self-annealed before its analysis.

Analysis of resistance to RNAase

RNAase resistance was measured by incubation of RNA in 0.3 M-NaCl/10 mM-EDTA/10 mM-Tris/HCl (pH 8) for 2 h at 37°C in the presence of 100 µg of RNAase A/ml and 150 ng of RNAase T1/ml (RNAases were from Sigma, St. Louis, MO, U.S.A.). Residual trichloroacetic acid-precipitable RNA was considered as double-stranded (Pays, 1976). Complete hydrolysis by RNAases was obtained by incubation in low-salt buffer.

Purification of dsRNA sequences

Total RNA was incubated in high-salt buffer with RNAases as described above, or with *Aspergillus* nuclease S1 (EC 3.1.4.-) under conditions already defined (Pays & Ronsse, 1975), then treated with three 100 µg portions of *Tritirachium* proteinase K/ml (EC 3.4.21.-; from Merck, Darmstadt, Germany) for three successive periods of 20 min at 37°C in the presence of 1% sodium dodecyl sulphate. Purified dsRNA was precipitated by addition of 2 vol. of ethanol for 24 h at -20°C, with unlabelled 4S RNA as carrier. dsRNA prepared in this way remains fully trichloroacetic acid-precipitable after incubation for 2 h or more in low-salt buffer at 37°C, or heating for 5 min at 100°C. It thus seems that no residual RNAase activity remains under these conditions.

Base-composition analysis

The base composition of RNA was determined by elution with a linear 0-0.17 M-HCl gradient of a column (45 × 1 cm) of AG1 resin (X2; Cl⁻ form),

after acid hydrolysis (in 1 M-HClO₄) for 48 h at 37°C. Under these conditions, no significant ³H exchange with water was observed (Evans *et al.*, 1970). Similar results were obtained by performing alkaline hydrolysis (in 0.5 M-KOH for 15 h at 30°C).

Hydroxyapatite fractionation

Hydroxyapatite (DNA grade; from Bio-Rad Laboratories, Richmond, CA, U.S.A.) was washed by decanting in 10 mM-sodium phosphate buffer, pH 6.8, and 0.5 ml of wet paste was poured into 3 ml syringes stoppered with a disc of GF/C glass-fibre filter. RNA (1 ml in 10 mM-sodium phosphate) was passed under pressure through the column, then rinsed with 2 ml of 10 mM-sodium phosphate. Release of single-stranded sequences was achieved by elution with three 2 ml portions of 0.12 M-sodium phosphate; double-stranded sequences were collected by elution with three 0.5 ml portions of 0.4 M-sodium phosphate. Unless stated otherwise, the temperature of elution was 50°C. The fractions, adjusted to 2 ml with water, were counted for radioactivity in vials containing 18 ml of 33% (v/v) Triton X-100 in Omnifluor (4 g/litre of toluene; from New England Nuclear Corp., Boston, MA, U.S.A.). With this procedure, quenching was identical in all fractions.

Polyacrylamide-gel electrophoresis

Electrophoresis of RNA was performed by the method of Loening (1967) in 10% (w/v) acrylamide gels with 0.2% sodium dodecyl sulphate. Gels were 9.4 cm long, with an upper 2.7% (w/v) acrylamide gel, 0.5 cm long. Electrophoresis was continued for 4 h at 25°C at 6 mA/tube. Bromophenol Blue and 4S and 5S bacterial RNA were used as markers. Gel slices (about 2 mm in length) were dissolved and counted for radioactivity in 5% Soluene (from Packard, Downers Grove, IL, U.S.A.) in Omnifluor.

Sephadex chromatography

RNA, in 0.5 ml of 0.3 M-NaCl/10 mM-EDTA/10 mM-Tris/HCl (pH 8), was fractionated by elution on a column (75 cm × 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 µl samples in 33% Triton X-100 in Omnifluor.

Results

Base composition

The dsRNA pieces synthesized *in vitro* by rat liver RNA polymerase B on chromatin were rich in cytidine (Pays, 1976). By using three labelled nucleotides (³H-labelled ATP, CTP and UTP, or GTP,

CTP and UTP) in the presence of all four unlabelled nucleotides, I have now obtained evidence for a symmetrical and (G+C)-rich base composition; purified dsRNA contained 31.4% CMP, 18.7% AMP, 19.2% UMP and 30.7% GMP, whereas total RNA had the base composition 26.3% CMP, 24.4% AMP, 23.4% UMP and 25.4% GMP. Total RNA synthesized with *E. coli* RNA polymerase was found to contain 28.7% CMP, 18.9% AMP, 23.6% UMP and 28.8% GMP, and the dsRNA sequences contained 31.9% CMP, 18.8% AMP, 17.4% UMP and 31.9% GMP. The dsRNA molecules synthesized on chromatin by the two polymerases thus exhibit the same base composition, although total RNA transcripts seem to be different in the two cases (in particular, transcripts by *E. coli* polymerase are poorer in adenine).

A large fraction of the transcripts contains one double-stranded sequence

Chromatin transcripts were analysed for their content of double-stranded sequences by two methods, namely elution on hydroxyapatite or RNAase treatment in high salt (see the Methods section). Provided that the transcripts were not heated above 50°C, about 70% of all molecules were bound to hydroxyapatite in 0.12M-sodium phosphate buffer as if they were double-stranded. However, this fraction was only 30% resistant to RNAase in high salt, indicating that it contained only 30% dsRNA; the sequences sensitive to ribonuclease treatment (70%) are probably covalently linked to the double-stranded segments. However, the fraction not bound to hydroxyapatite in 0.12M-sodium phosphate (30% of total RNA) also showed significant resistance (20%) to RNAase in high salt, and thus also contained dsRNA. The reason why these dsRNA sequences did not bind to hydroxyapatite seems to be their small size. Indeed, analysis on 10% (w/v) polyacrylamide gels revealed that this fraction contained only sequences smaller than the 4S tRNA marker, with dsRNA fragments of less than 20 nucleotides (E. Pays, unpublished work). One can thus assume that the majority of the RNA molecules synthesized *in vitro* on chromatin contain a double-stranded sequence.

Double-stranded transcripts are intramolecularly base-paired

When the chromatin transcripts were eluted from hydroxyapatite at different temperatures, the denaturation curve of their double-stranded sequences was obtained (Fig. 1, curve 1, ■). The T_m ('melting' temperature) was 72°C. The same T_m was found when dsRNA sequences, purified from whole transcripts as described in the Methods section, were

Fig. 1. Thermal denaturation of dsRNA from chromatin transcripts by rat liver RNA polymerase

Curve 1 (■) shows the denaturation of dsRNA in whole transcripts as measured by hydroxyapatite fractionation. The RNA was heated for 5 min at different temperatures when bound to hydroxyapatite in 0.12M-sodium phosphate buffer, then eluted in 0.4M-phosphate at the same temperature. The fraction bound to the column in 0.12M-phosphate was considered as double-stranded and is expressed as percentage of retention on hydroxyapatite. Binding to hydroxyapatite in 0.12M-phosphate at 50°C (70% of total RNA) was taken as 100%. Points labelled 4 (□): as for curve 1, but in this case retention on hydroxyapatite was measured at 50°C, immediately after preheating of RNA at 80°C or 100°C in 0.01M-sodium phosphate buffer. Curve 2 (○): denaturation of dsRNA purified from whole transcripts, as measured by analysis of resistance to RNAase in high salt after heating. The RNA was heated for 5 min at different temperatures in 10mM-EDTA/10mM-Tris/HCl, pH8, then its percentage resistance to RNAase in 0.3M-NaCl was determined (see the Methods section). Resistance to RNAase at 25°C (96% of input RNA) was taken as 100%. Curve 3 (●): denaturation of dsRNA sequences included in whole transcripts, measured as for curve 2. Resistance to RNAase at 25°C (31% of total RNA) was taken as 100%.

assayed for resistance to RNAase digestion in high salt after thermal denaturation in 10mM-EDTA/10mM-Tris/HCl (pH8) (Fig. 1, curve 2, ○). In contrast, the denaturation of whole RNA molecules, measured by resistance to RNAase after heating in the same way, appeared to be slower and incomplete (about 50% at 100°C) (Fig. 1, curve 3, ●). It thus seems that the purification of dsRNA from whole RNA molecules, by hydrolysis of single-stranded sequences, is needed to obtain their complete denaturation by heating. Presumably the single-stranded piece linking the two complementary strands of dsRNA in intact molecules permits their very fast renaturation on cooling; as a consequence, some double-stranded regions of intact molecules could escape RNAase digestion even after being heated at 100°C in low salt.

That the complementary strands of dsRNA are intramolecularly base-paired is further suggested by the following experiments.

(1) If RNA is heated at 80 or 100°C, then quickly cooled to 50°C and chromatographed on hydroxyapatite (see the Methods section), I observed only a 20% decrease in RNA binding to the column in 0.12M-sodium phosphate (Fig. 1, points labelled 4, □). By contrast, when elution on hydroxyapatite is performed at 80 or 100°C, the large majority of the RNA molecules do not bind to the column in 0.12M-phosphate (Fig. 1, curve 1). This confirms that all the dsRNA sequences are effectively denatured at 100°C in low-salt buffer, but 80% of it can readily reanneal on cooling, as indicated by its binding to hydroxyapatite at 50°C. Since this effect is totally independent of the RNA concentration, it seems that the renaturation of a large fraction of the double-stranded pieces in whole transcripts follows first-order kinetics, as would be expected if these segments are in the same molecule.

(2) The electrophoretic mobility of purified dsRNA sequences changes markedly after denaturation (60°C in 75% v/v, formamide) (Fig. 2a), but under the same conditions of heating, whole transcripts (untreated by RNAase in high salt) remain exactly at the same place in the gel (Fig. 2b). Moreover, after heating of the whole molecules at 60°C in 75% formamide, a large fraction of total dsRNA sequences can be purified, and migrate in the gel as does unheated dsRNA (Fig. 2c). This means that in intact molecules the large majority of dsRNA complementary strands remain linked together even in the conditions of denaturation of dsRNA, and can be rapidly renatured.

In contrast with these observations, I obtained only a maximum of 9% renaturation of whole transcripts when measuring it by RNAase resistance in high salt after heating at 80 or 100°C for 5 min in 40% (v/v) formamide, followed by annealing for 5 min or more in 0.3M-NaCl/40% formamide at 37 or 50°C [the denaturation percentage of whole transcripts was 58% before annealing, and 49% after annealing at a maximum R_0t (mol of nucleotides $\times$ time of annealing) of about 10^{-5} M $\cdot$ s]. Only 15.5% of the denaturated sequences can thus quickly recover their resistance to RNAase in high salt after 'melting'. The reasons for this discrepancy are considered in part 4 of the Discussion section.

Thermal stability of dsRNA sequences is size-dependent

The dsRNA sequences of chromatin transcripts are fractionated in Sephadex G-100 in high salt, but are excluded from the same column in water (Fig. 3). They thus appear to be in a more compact configuration in high salt. Under these conditions, 35–45% of them appear to be larger than the 4S tRNA marker (Fig. 3b). Similar results were obtained by electrophoresis on 10% (w/v) polyacrylamide gels (results not shown here; but see Fig. 7).

We have isolated on Sephadex G-100 different dsRNA subfractions, the denaturation properties of which were analysed separately. The thermal stability of the double-stranded pieces is size-dependent; the T_m is higher for the larger dsRNA sequences (Fig. 4). This effect is not due to a difference in base composition (61.4% G+C for the largest sequences, and 64.3% for the smallest). It could mean that the

Fig. 2. Denaturation of dsRNA sequences, as revealed by 10% (w/v) polyacrylamide-gel electrophoresis

In (a), purified dsRNA sequences of about 5S (fraction between a and b of Fig. 3b) were analysed after heating for 5 min at 25°C (■) or at 60°C (○) in 75% formamide/10 mM-EDTA/10 mM-Tris/HCl (pH 8), followed by precipitation with ethanol. Gels were sliced into 2 mm sections, which were counted for radioactivity as described in the Methods section. In (b), the same analysis was performed, but on a whole RNA fraction of about the same size as that of purified dsRNA. (c) Shows electrophoresis of dsRNA sequences isolated after treatment of the whole RNA transcripts at 25°C (■) or at 60°C (○) in the same conditions as above. The location of 4S tRNA marker is indicated by one arrow; the position of the Bromophenol Blue dye is indicated by the double arrow. Heating in 75% (v/v) formamide was designed to denature all dsRNA at the lowest temperature possible, avoiding thermal breakages which could interfere in such a study. Similar results were obtained by heating at 80°C in 40% formamide.

Fig. 3. Chromatography of purified dsRNA sequences on Sephadex G-100, eluted in water (a), or in 0.3M-NaCl (b). Elution of dsRNA (■) on Sephadex G-100 was as described in the Methods section. Fractions of volume 1.5 ml were collected. ○, Position of the 4S RNA marker. The void volume ceases at fraction 13 (arrow). dsRNA molecules as well as tRNA are excluded as a sharp peak when elution was with water (fractions 14–17 in a). The second peak in (a) (fractions 40–47) gives the position of the nucleotides produced by the RNAase digestion in high salt, in an experiment where no precipitation with ethanol was performed after the hydrolysis of single-stranded RNA sequences. In (b), fractions 16–20, 24–28 and 31–35 were pooled separately as fractions a, b and c respectively, and their thermal stability was analysed as described in Fig. 4.

smallest dsRNA sequences contain more mismatched base-pairs. Since a difference of 15°C in T_m represents a base-substitution frequency of approx. 10% (Ullman & MacCarthy, 1973), the smallest dsRNA sequences could contain about 13% mispaired bases by comparison with the largest dsRNA species. Other possibilities are that the longer dsRNA sequences possess a tertiary structure stabilizing them against thermal denaturation, or that the size-dependence of T_m could be abolished above the minimal stable length estimated for a nucleic acid duplex (about 20 nucleotides; Walker, 1969).

The whole RNA transcripts were fractionated on Sephadex G-100, and their thermal stability was analysed as described above for purified dsRNA species (Figs. 5 and 6). A size-dependence was observed for the thermal stability of dsRNA segments included in whole transcripts, but denaturation was always found incomplete, even for the smallest

Fig. 4. Thermal denaturation of different dsRNA fractions from Sephadex G-100 chromatography (a) Fractions a (■), b (○) and c (●) of Fig. 3(b) were heated for 5 min at different temperatures in 10mM-EDTA/10mM-Tris/HCl, pH8, then analysed for RNAase resistance in high salt. (b) Denaturation curve of total dsRNA sequences.

RNA sequences. This size-dependence suggests that some relationship exists between the size of whole molecules and that of their double-stranded pieces. This was verified by size analysis on polyacrylamide gels of the dsRNA molecules isolated from the fractionated whole transcripts (Fig. 7). It was found that not only the size, but also the proportion of dsRNA, is proportional to the size of the whole transcripts. For the transcripts synthesized by rat liver polymerase, the proportion of dsRNA varied from 50.6% for the largest RNA pieces, to 16.3% for the smallest, whereas the corresponding values for transcripts by *E. coli* polymerase were 34.1 and 5.4% (Fig. 5).

Fig. 5. Fractionation on Sephadex G-100 of whole chromatin transcripts synthesized by rat liver (■) or *E. coli* (Δ) RNA polymerases

Elution was in 0.3M-NaCl/10mM-EDTA/10mM-Tris/HCl (pH8). Fractions of volume 1.5ml were collected. The positions of *E. coli* 5S and 4S RNA markers, eluted in the same conditions, are shown. Fractions 13–16, 20–22 and 25–28 were pooled separately as fractions a, b and c respectively, and analysed as described in Fig. 6 and Fig. 7. The percentage of dsRNA sequences was determined in some fractions of rat liver (●) or *E. coli* (□) transcripts.

Fig. 6. Denaturation curves of dsRNA sequences included in whole chromatin transcripts fractionated on Sephadex G-100

Fractions a (□, ■) and c (Δ, ▲) were obtained from Sephadex chromatography of rat liver (□, Δ) or *E. coli* (■, ▲) polymerase transcripts (see Fig. 5). Their percentage of resistance to RNAase in high salt was measured after incubation for 5 min at different temperatures in low salt, as described for Fig. 4. Resistance at 25°C was taken as 100%; it was actually 50.6% or 29.1% for a, and 16.3% or 7.5% for c, in each case for rat liver or *E. coli* polymerase transcripts respectively.

Comparison of the stability of dsRNA sequences synthesized by either rat liver or *E. coli* RNA polymerase

Chromatin transcripts by rat liver RNA polymerase contain a greater proportion of double-stranded segments than do those synthesized by a bacterial polymerase (Fig. 5). Moreover, it seems that the dsRNA sequences synthesized by the rat liver enzyme are more stable than the bacterial ones, as indicated by differences in their resistance to RNAase degradation as a function of either temperature (Fig. 8) or salt concentration of the medium (Fig. 9). The 'melting' temperature of dsRNA synthesized by the rat liver polymerase is 5–10°C higher than that of dsRNA synthesized by *E. coli* polymerase, for similar RNA size and base composition. The salt concentration needed for stabilization of dsRNA synthesized by the rat liver polymerase is 0.32–0.34M-NaCl, whereas it is 0.40–0.50M-NaCl for transcripts by bacterial polymerase.

The stabilization by salt appears to be independent of the size of RNA, but it is not the case for the 'melting' temperature. This could indicate that the differences in T_m between different size classes of dsRNA species are not only due to different extents of mismatching as a function of size, but rather that the minimal stable length of RNA duplexes (versus temperature) could be significantly more than 20 base-pairs (Walker, 1969).

When similar investigations were carried out on RNA synthesized on pure DNA, we found the same T_m and salt concentration needed for stabilization of dsRNA transcripts by either rat liver or *E. coli* RNA polymerase (results not shown). The NaCl concentration needed for stabilization of dsRNA synthesized on pure DNA was 0.38–0.48M, as for dsRNA synthesized on chromatin by *E. coli* polymerase, but higher than that measured for chromatin transcripts by liver polymerase (0.32M-NaCl).

Discussion

(1) The synthesis of RNA *in vitro* was performed under conditions limiting elongation of RNA chains while optimizing initiation specificity (low RNA polymerase/DNA ratio; low ionic strength; un-sheared template) (Cox, 1973; Story & Wheldrake, 1975; Tsai *et al.*, 1975). The short RNA sequences obtained under these conditions could be preferentially transcribed on palindromic DNA sequences, since they are particularly rich in double-stranded 'hairpins'. This could be compared with the results of Shen & Hearst (1976), showing the presence of palindromes in or near promoter sites for RNA polymerase in the DNA of bacteriophage fd.

(2) Since, for the same size, a greater proportion of dsRNA sequences was measured in chromatin

Fig. 7. Content and size of dsRNA sequences from different fractions of chromatin transcripts synthesized by rat liver RNA polymerase B, as revealed by electrophoresis on 10% (w/v) polyacrylamide gels

RNA transcripts were fractionated on Sephadex G-100 in 0.3M-NaCl, as described in Fig. 5. Fractions a, b and c from chromatography in Fig. 5 were electrophoresed in (a), (b) and (c) respectively, after incubation for 2 h at 37°C in high-salt buffer in the presence (○) or absence (■) of RNAses A+T1, followed by proteinase K treatment (see the Methods section). The position of the markers is indicated as for Fig. 2.

Fig. 8. Thermal stability of purified dsRNA sequences from chromatin RNA transcripts synthesized by either *E. coli* (a) or rat liver (b) RNA polymerase

Purified dsRNA sequences were fractionated according to their size as described for Fig. 3. Fractions 16–21 and 26–31 were pooled as fractions a and b respectively, and denatured as described for Fig. 4. The T_m is indicated by one arrow.

transcripts than in RNA synthesized on DNA, and with homologous RNA polymerase than with bacterial, some specificity could be associated with their presence in the initial transcription products. Moreover, we found reproducibly that the dsRNA sequences synthesized on chromatin by the homologous RNA polymerase are more stable than those synthesized by *E. coli* polymerase on chromatin, or those synthesized on pure DNA by either of the two polymerases. This was observed in homogeneous fractions of about the same size and base composition, and with RNA polymerases prepared freshly or stored for different periods of time. The most plausible explanation is that dsRNA synthesized on chromatin by the homologous RNA polymerase is less mismatched than the other dsRNA preparations.

Tuffet & Huguet (1975) noted that dsRNA molecules in transcripts of wheat DNA by *E. coli* RNA polymerase are only stabilized in 0.5M-NaCl, as in my experiments with rat DNA. Moreover, Crain & Saunders (1976) also found a greater percentage of double-stranded RNA in human chromatin transcripts synthesized by *E. coli* polymerase, by comparison with pure DNA transcripts.

(3) Synthesis of dsRNA occurs preferentially at low ionic strength, on chromatin as well as on pure DNA. In 100 mM-(NH₄)₂SO₄, synthesis of dsRNA by rat liver polymerase was indeed decreased to 20% by comparison with the synthesis in 5 mM-(NH₄)₂SO₄; with *E. coli* polymerase, the dsRNA synthesis was decreased to about 50% (E. Pays, unpublished work). Since the double-stranded structures of DNA are stabilized at high ionic strength, it is possible that they cannot be sufficiently denatured to permit their transcription in high-salt buffer *in vitro*. Indeed, the synthesis of dsRNA sequences in high salt is poorer with the eukaryotic polymerase than with the bacterial enzyme; this could be related to the fact that *E. coli* polymerase transcribes double-stranded DNA more efficiently, whereas the rat liver polymerase prefers denatured DNA (E. Pays, unpublished work).

(4) The results presented here are compatible with the presence of a single-stranded link between the

Fig. 9. Stabilization by salt of dsRNA sequences in chromatin RNA transcripts synthesized by either *E. coli* (a) or rat liver (b) RNA polymerase

Resistance to RNAase was assayed as a function of NaCl concentration on chromatin transcripts fractionated as in Fig. 5. Fractions 14–19 and 25–30 were pooled as fractions a and b respectively. The NaCl concentration corresponding to one-half of that needed for maximal stabilization of double-stranded sequences is indicated by a broken line (----). The same kind of result was obtained when assaying stabilization by salt in homogeneous fractions of purified dsRNA sequences fractionated as described for Fig. 3, although in this case the difference between (a) and (b) was always smaller.

methods that we have used. On hydroxyapatite, the extent of reannealing was estimated to be about 80% (Fig. 1, points labelled 4), but it was about 50% when measured by resistance to RNAase digestion in high salt (Fig. 1, curve 3). However, by using the last method of measurement, no extensive renaturation (maximum 15.5%) was observed in the denatured fraction of whole transcripts, even after prolonged incubation. This suggests either that some of the complementary sequences could be easily separated after heating, or that the intermediary piece might be sufficiently long to prevent a rapid renaturation of a fraction of dsRNA. It could also be supposed that during a rapid renaturation, extensive mismatching of base-pairs occurs (Darzynkiewicz *et al.*, 1975), so that the extent of renaturation appears to be different when either the 'RNAase' or the 'hydroxyapatite' method is used for measuring the proportion of double strand.

(5) 'Melting' of dsRNA was found to be size-dependent, but stabilization by salt appears to be independent of size. In view of the latter observation, it is unlikely that some differential mismatching of base-pairs as a function of RNA size could alone explain the differential thermal stability of dsRNA as a function of its size. Since the smallest and largest dsRNA subfractions have a similar base composition, we conclude that the size-dependence of T_m , for dsRNA synthesized *in vitro*, could be abolished for sequences longer than the minimal stable length estimated for double-stranded DNA (about 20 nucleotides; Walker, 1969). Concerning this point, the thermal stability of commercially available short oligonucleotides is far below what has been observed with the short duplexes in naturally occurring RNA species (Gralla & Crothers, 1973a,b). Double-stranded sequences synthesized *in vitro*, whether free or not of the relatively short neighbouring single strands, could thus be less stabilized than similar sequences in 'natural' RNA species.

(6) The length of dsRNA sequences increases with the size of the whole transcripts. This observation is at present difficult to explain, but could be expected if some long complementary sequences are separated by a long intermediary single-stranded segment; then the longer the transcript is, the greater will be the proportion of dsRNA and its size in the transcript. This observation could be better explained by assuming that dsRNA arises from symmetrical transcription of both strands of DNA. However, results presented above (Figs. 1 and 2) strongly suggest that dsRNA is intramolecularly base-paired.

(7) The huge amount and (G+C)-rich base composition of double-stranded sequences in chromatin transcripts suggest analogy with rRNA. It is, however, improbable that rRNA or tRNA are synthesized on the chromatin under the present conditions with purified nucleoplasmic RNA polymerase B, since this

complementary strands of dsRNA, since a significant fraction of dsRNA can readily reanneal after 'melting'. There are, however, discrepancies between the extents of renaturation measured by the two

RNA synthesis *in vitro* is inhibited by about 95% with 0.1 μ g of α -amanitin/ml, a specific inhibitor of nucleoplasmic RNA polymerase. Endogenous RNA polymerase activity of chromatin (which is only about 50% inhibited by α -amanitin) represents no more than 13% of total RNA synthesis, and does not result in the production of double-stranded sequences (Pays, 1976). However, the possibility that rRNA or tRNA are preferentially synthesized at low ionic strength by RNA polymerase B on chromatin *in vitro* cannot be completely ruled out.

I am grateful to Professor J. Brachet, Dr. R. Kram, Dr. C. Thomas and Dr. F. Hanocq for helpful discussions and comments on the manuscript. The excellent technical assistance of Mrs. N. Herremans is also acknowledged.

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