Hybridization of pigeon globin messenger RNA with complementary DNA synthesized in vitro by reverse transcription: influence of the homopolymeric regions

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Received 3 June 1975

ABSTRACT

The kinetics of hybridization of pigeon globin messenger RNA with complementary cDNA synthesized by means of AMV reverse transcriptase is complex. Addition of poly A or poly U in excess to the reaction mixture normalized the kinetics. It is concluded that association of the complementary homopolymeric regions of mRNA and cDNA accelerates the complex formation between heteropolymeric sequences in a fraction of the molecules.

INTRODUCTION

As known messenger RNAs with polyadenylic sequences at their 3' end may be transcribed by RNA-directed DNA-polymerase in the presence of oligothymidylate primer¹⁻⁷. It has been shown experimentally⁸ that in the course of reverse transcription poly T sequences appeared in cDNA probably due to transcription from poly A portions of mRNAs.

The aim of this work was to elucidate the influence of poly T and poly A regions in the cDNA and mRNA respectively on their hybridization kinetics.

MATERIALS AND METHODS

³H dGTP was from Amersham, [³H]TTP was obtained from Prof. P. Langen and Prof. G. Vormum (DDR) via "Revertase" project. Oligo(dT)₁₀ was from PL Biochemicals and actinomycin D from Reanal. DE-52 and P-II ion-exchange celluloses from Whatman were used. Takadiastase was obtained from Sankyo.

9 S mRNA was isolated from reticulocyte polysomes; reticulocytes were obtained from phenylhydrazine injected pigeons⁹. After cell lysis polysomes were pelleted by centrifugation and used for RNA isolation¹⁰. 9 S fraction was isolated after chromatography of polysomal RNA on the poly U-column as described by Sheldon et al.¹¹. At variance with the published procedure¹¹

the column after RNA sorption was washed with eluting buffer at 4°C and the elution was performed at 40°. This modification leads to increase in the overall yield of mRNA and reduces the rRNA impurities in the 9 S fraction. After additional centrifugation in sucrose concentration gradient RNA obtained sedimented as a homogeneous peak with a maximum at 9 S (Fig. 1).

Fig. 1. Sucrose gradient centrifugation of the polysomal RNA from pigeon reticulocytes, (a); its fraction eluted from poly U-cellulose column, (b); and 9 S fraction taken from (b) as shown by arrows, (c).

5-20% sucrose gradient in 0.01 M K-acetate (pH 5.2) - 0.1 M NaCl (Hitachi, RPS-40, 40 000 rpm, 5 h, 2°C).

Reverse transcriptase was isolated from avian myeloblastosis virus as described in ¹² with small modifications ⁴. Transcription reaction was conducted in the presence of oligo(dT) and actinomycin D for 1 hour at 37°. DNA product was treated with alkali and precipitated by ethanol after the addition of 50 µg/ml of yeast tRNA. [³H]dGTP and [³H]dTTP with specific activities of 1500 cpm/pmol were used as labeled precursors. In all cases besides those mentioned especially [³H]GTP was used as a precursor.

The synthesized product (cDNA) had sedimentation coefficient 7 S, its buoyant density in Cs₂SO₄ was equal to 1.470 g/cm³ as already described ⁴.

Hybridization was conducted in 0.24 M phosphate buffer (PB) containing equimolar amounts of Na₂HPO₄ and NaH₂PO₄ with 0.1% sodium dodecyl sulphate in the final volume of 15 µl. cDNA, mRNA (ratio 1 : 10) and other ingredients were mixed at 0°C or at 18-20°C and then annealed in sealed capillaries. After annealing the hybridization mixtures were diluted 50 times

with a buffer containing 0.03 M Na acetate pH 4.5, 0.02 M NaCl and 0.03 mM ZnSO_4 ; denatured DNA was added (25 $\mu\text{g}/\text{ml}$) and the mixture was treated 90 min at 37° with S_1 nuclease purified from takadiastase¹³. Undigested material was precipitated with TCA added to a final concentration of 7% (100 μg chick DNA was added as a carrier before TCA) and collected on membrane filters. Radioactivity was measured in toluene scintillator.

RESULTS

About 95% of the synthesized cDNA is sensitive to S_1 nuclease (Table I). After the self-annealing for 2 hours under hybridization conditions the amount of S_1 resistant cDNA increases to 10–12%. Similar increase is observed after the annealing of cDNA with 28 S rRNA from reticulocyte polysomes.

Table I.

The effect of annealing upon the resistance of the cDNA to S_1 nuclease

Annealing*	cDNA resistant to S_1 nuclease, %
Without annealing	5–6
Self-annealing	10–12
With rRNA	12–14
With mRNA	90–95

* cDNA (1500 cpm) was annealed for 2 hours at 67° and treated with the enzyme (see Methods).

To elucidate whether poly A sequences were copied in our experiments, cDNA synthesized in the presence of $[^3\text{H}]\text{dTTP}$ was annealed with the excess of poly A and treated with S_1 nuclease. Up to 40% of $[^3\text{H}]\text{dTTP}$ incorporated into cDNA is protected from S_1 nuclease after annealing with poly A (Table 2). Under the same conditions the sensitivity of $[^3\text{H}]\text{dGMP}$ labeled product to S_1 nuclease remains unchanged. The latter observation proves that there is no unspecific binding of added poly A with cDNA heteropolymeric regions.

We conclude from these results that cDNA synthesized in our experiments contained sufficiently long poly dT sequences able to form hybrids with poly A and to become resistant to S_1 nuclease. As already shown in Table 1 after hybridization with

saturating amounts of pigeon globin mRNA 90-95% of the cDNA becomes resistant to S_1 nuclease. In addition, as shown by us earlier ¹⁴ hybrids of cDNA with mRNA could be separated from single-stranded DNA by chromatography on hydroxyapatite (HAP); melting of hybrids immobilized on HAP columns has shown that their heat stability is comparable to that of fragmented native E.coli DNA. This corresponds to a GC content of ~50% and is in agreement with the data on GC content of globin mRNA from several animal species ^{15,16}.

Table 2

The effect of annealing with poly A on the resistance of cDNA* to S_1 nuclease

Labeled precursor	poly A	cDNA resistant to S_1 nuclease, %
$[^3\text{H}]\text{dGTP}$	-	5.5
	+	5.1
$[^3\text{H}]\text{TTP}$	-	5.1
	+	45.0

* cDNA (1500 cpm) and poly A (50 $\mu\text{g}/\text{ml}$) were heated for 10 min at 60° and then cooled during 1 hour at room temperature.

If the kinetic curve of hybridization between cDNA and mRNA (Fig. 2) is plotted in the linear scale using the equation for the second order reaction kinetics (Fig. 2, insert) one can see that the initial phase of the reaction proceeds at a very high rate. This phenomenon demonstrates that hybridization kinetics between cDNA and mRNA is complex suggesting the presence of components with very different reaction rate constants. At least two components (or fractions) can be discerned.

One of them containing about 70% of the labeled material is hybridized at a moderate rate and the course of reaction is linear in the chosen coordinates. The reaction rate constant of the other, smaller fraction is so high that the course of its hybridization cannot be adequately described with the time scale used in this experiment. Even after 1 minute at 20° about 30% of the cDNA becomes resistant to the S_1 nuclease (Fig. 3). Further incubation for 2 hours at this temperature does not result in

Fig. 2. Kinetic curves of hybridization between cDNA and mRNA without ($\Delta-\Delta-\Delta$) and with 0.5 $\mu\text{g}/\text{tube}$ of poly A (O-O-O) or poly U ($\bullet-\bullet-\bullet$)

The annealing temperature 67° . Zero time - cDNA + mRNA at 2-4° 5-10 sec. The insert represents a second-order rate plot for the data of Fig. 2: $\Delta-\Delta-\Delta$, O-O-O, $\bullet-\bullet-\bullet$ - after the subtraction of 20% resistance observed at zero time; $\blacktriangle-\blacktriangle-\blacktriangle$ - after additional subtraction of 12% resistance due to the interaction of poly T and poly A sequences. R - relative proportion of cDNA in hybrids.

Fig. 3. Kinetic curves of hybridization between the cDNA and mRNA at 20° without addition (O-O-O) and with preaddition ($\bullet-\bullet-\bullet$) of poly A.

any marked increase of hybridization. One can conclude that the bulk of cDNA is not hybridized under these conditions and can be separated from the rapidly hybridizing fraction.

The presence of the fast hybridizing component may be very well revealed at 20° when the behaviour of only fast hybridizing fraction is expressed (Fig. 3).

The high rate of hybridization reaction between the fraction of cDNA and mRNA may be due to the interaction of their poly T and poly A sequences. If so this contribution of homopolymeric regions can be circumvented when the hybridization is conducted in the presence of excessive amounts of cold poly A or poly U. As shown in Fig. 2 the addition of each of these homopolymers to the reaction mixture results in similar changes in the course of hybridization: part of the fast component disappears. The identical effects of poly A and poly U imply that both of these homopolymers affect one and the same mechanism although from different sides. Addition of the excess of poly A decreases the rapidly hybridizable fraction of cDNA by about 1/3, or 10-12% of the total DNA.

Fig. 4 shows thermal elution from hydroxyapatite for mRNA-cDNA hybrids formed at 67° and 20°. It is noteworthy that the hybrids formed at 20° have the same high thermal stability as those obtained in conditions optimal for hybridization of heteropolymers.

The excess of poly A added does not completely prevent the increase of the cDNA resistance to S_1 nuclease hydrolysis when it is hybridized to mRNA under conditions described. About 15% of the cDNA (after the subtraction of 5% characteristic for initial non-annealed cDNA) becomes resistant to S_1 nuclease even in the presence of poly A. At the same time the resistance after annealing with rRNA does not exceed 1-2% if the self-annealing of the cDNA in the same conditions is subtracted. At present we cannot explain why 15% of the cDNA becomes resistant to S_1 nuclease after brief incubation with mRNA at low temperatures in the presence of poly A.

If to subtract from the hybridization data 15% and 5% resistance which are characteristic for non-annealed cDNA the hybridization reaction between mRNA and cDNA in the presence of

poly A or poly U follows the second order reaction rate (Fig. 2, insertion). Hybridization of the mRNA with cDNA without poly A or poly U additions proceeds in the same way if 12 % of hybrids formed due to the interactions of homopolymeric portions of the molecules have been subtracted from the curve (Fig. 2).

However as may be seen from the next table 3 these data do not mean that only 12% of the cDNA molecules do contain poly T sequences. When cDNA preparation has been passed through poly A-cellulose column approximately half of the molecules were bound by the column. Independently it was shown that under conditions used only poly T containing sequences are retarded by the poly A-column. cDNAs containing poly T sequence are able to form certain amount ($\sim 37\%$) of hybrids at 20° and formation of these hybrids is suppressed by the presence of poly A (Table 3).

Contrary to that cDNA-mRNA hybridization sensitive to the presence of poly A was not observed at 20° with cDNA fraction without poly T (non-adsorbed on the poly A-column). However both cDNA fractions are able to hybridize at 67° . From table 3 one may see also that not all poly T containing cDNA molecules hybridise with an increased rate although the proportion of these molecules in this cDNA fraction is higher then in total cDNA. Probably the homopolymer effect on the hybridization of heteropolymeric sequences may be realized only in those cases where the latter are arranged by chance in complementary positions.

Table 3.

S_1 nuclease resistance (in %) of the cDNA after annealing with or without globin mRNA and poly A

cDNA	Annealing		
	without mRNA	with mRNA +poly A	+tRNA
1 total cDNA	4.6	14.7	22.7
2 cDNA fraction not retained on the poly A-column	9.0	15.6	16.2
3 cDNA fraction, retained on the poly A-column	6.2	19.7	37.0

[^3H]dCMP-DNA (sp. act. $0.5 \cdot 10^6$ cmp/ug) was fractionated on poly A-cellulose column ¹¹. The fraction retained on the column was eluted at 50° with tris $\cdot$ HCl, 0.01 M (pH 7.5). Both fractions were

treated with 0.33 M KOH at 37° during 18 h and annealed (30 min, 20°, 0.24 M phosphate buffer, pH 7.0) in aliquotes of 15 μ l. Yeast tRNA in the same amount was used as a control in those tubes where no poly A was added. The value of $S_{1/2}$ -nuclease resistant fraction measured with cDNA alone without annealing was subtracted from all the data. All cDNA fractions were able to hybridize with mRNA in optimal conditions (67°, 2 h) with 75-85% efficiency.

Fig. 4. Thermal elution profiles of cDNA from HAP columns after annealing with mRNA.

Δ-Δ-Δ - for 2 hours at 67° (90% of binding with HAP), O-O-O - for 1 min at 20° in the absence poly A (30% of binding with HAP), ●-●-● - for 1 min at 20° in the presence of poly A (19% of binding with HAP). After the annealing cDNA was adsorbed onto HAP in 0.12 M PB at 72° and then eluted with 0.06 M PB at different temperatures. The cDNA remaining on the column at 96° was eluted with 0.4 M PB.

DISCUSSION

The results demonstrate that the simultaneous presence of poly A in mRNA and poly T in the cDNA accelerates the hybridization of heteropolymeric sequences in a fraction of molecules and is responsible for a complex pattern of the hybridization reaction. Probably rapid interaction of homopolymeric region provided a "nucleation" facilitating more rapid interaction of heteropolymeric sequences. Hybridization of the main part of the cDNA is however not accelerated as seen from Fig. 2. One may assume that poly A - poly T hybridization takes place in all cases but the exact fit of heteropolymeric regions is achieved only in a limited fraction of interacting molecules in the given experimental conditions. It is also possible that these molecules contain short poly T sequences insufficient to affect the hybridization rate of heteropolymeric parts.

The interpretation according to which the complex kinetics of hybridization is accounted for interactions between poly A and poly T of mRNA and cDNA respectively is supported by recently published data of Harrison et al.¹⁷. They have shown that if no poly T regions present in the cDNA the hybridization follows the second order reaction kinetics without fast-hybridizing fraction observed in this work.

Under the conditions of hybridization (0.36 M Na⁺, 67°) the formation of stable (poly A)·(poly T) complexes should not take place¹⁸. Nevertheless as follows from the above data their interaction facilitate the hybridization of the heteropolymeric regions.

Thus the hybridization of mRNA with cDNA containing a polythymidilate sequence is an unusual phenomenon. At present the kinetics of complementary base pairing as well as the physics of this process is more or less understood for both heteropolymeric and homopolymeric polynucleotides. However in the case described we deal with "chimeric" polynucleotide when the high rate of interaction of homopolymeric regions stimulate the hybridization of heteropolymeric parts of the molecules. Further work with this model may give a more deep insight in to the mutual influence of the different polynucleotide sequences neighbouring within one chain on the rate constant of their complex formation. This problem deserves special attention since in the eukaryotic genomes and in their transcripts similar alternating homo and heteropolymeric sequences may exist¹⁹⁻²¹.

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