Volume 2 number 7 July 1975 Nucleic Acids Research

Hybridization of polyuridylic acid to human DNA immobilized onto nitrocellulose filters

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Received 20 May 1975

ABSTRACT

The level of deoxyadenylate (dA) regions in human DNA was estimated from formation of poly(U)-poly(dA) triplexes on nitrocellulose filters that were RNAase resistant. The (dA) rich sequences were determined following mild ribonuclease treatment of the poly(U)-DNA hybrids (5 μg/ml at 25°C for 30 min), where as exhaustive ribonuclease treatment (5 μg/ml at 25°C for 6 hr) estimated the more (dA) pure sequences. The level of (dA) rich regions was 0.39 % of the DNA and for the more (dA) pure regions it was 0.07 %.

The (dA) regions were widely distributed throughout human DNA regardless of base composition or sequence repetition. However, a concentration of (dA) regions into main band CsCl gradient fractions of DNA and into repeated DNA was observed.

INTRODUCTION

The occurrence of poly(rA) sequences up to 200 nucleotides in length has been well established for eukaryotic mRNA[1-3]. In addition, oligo(U)[4,5] and oligo(A)[6] rich sequences of approximately 30 nucleotides have also been found in HnRNA from mammals. As a result, the question of whether these ribohomopolymeric regions were encoded from DNA or attached post-transcriptionally, has motivated the search for the corresponding deoxypolymeric stretches in DNA.

The only extensive quantitative studies of polyuridylic acid (poly U) hybridization to (dA) rich sequences in DNA were reported by Shenkin and Burdon[7,8] using hamster DNA and Bishop et al[9] using duck DNA. In both cases, the parameter studies for formation of poly(U)-DNA hybrids were carried out with both the poly(U) and either poly(dA) or DNA in solution. It was established[9] that a ribonuclease-resistant 2 poly(U)-poly(dA) triplex was formed with the annealing conditions used for poly(U)-DNA

hybridizations. In addition, the level of (dA) regions in DNA was difficult to quantitate in solution because the poly(U)-DNA hybrids were highly sensitive to ribonuclease digestion, presumably due to the presence of other deoxynucleotides within the hybridizing region[8].

Hybridization techniques involving DNA immobilized on nitrocellulose filters have the inherent advantage of comparing different DNA species or different fractions of the same DNA under identical conditions of ribonuclease treatment. Therefore, whether filter hybridization of poly(U) to DNA can be used effectively in quantitative studies of the (dA) regions in human DNA was determined. This was done to eliminate any subtle ribonuclease treatment differences that may influence the distribution pattern of (dA) regions among different fractions of human DNA.

There have been no detailed studies of the (dA) regions in human DNA. Molloy et al[5] have reported that oligo(U) isolated from HnRNA of HeLa cells hybridizes rapidly to HeLa DNA. Marshall and Gillespie[10] have mentioned that poly(U) complexes with 0.5 % of the human DNA but only 0.005 % of the hybrid is resistant to ribonuclease A. However, their data were unpublished and no further details presented. Therefore, it was also the purpose of this study to characterize the level of (dA) sequences present in human DNA and describe their distribution within the genome.

MATERIALS AND METHODS

Materials. The sources of (^{3}H)poly(U) were Miles Laboratories, Inc. (47.0 and 58.9 mCi/mMole monophosphate). Unlabelled poly(U), poly(dA), salmon sperm DNA, ribonuclease A (Bovine Pancreas) and alpha-amylase were purchased from Sigma chemical company. Pronase-P was the product of Kaken chemical company (lot no. 592045). The hydroxyapatite used was Bio Gel HTP from Bio-Rad Laboratories. Lymphocytes were isolated from the heparinized venous blood of normal human subjects as already described[11]. Normal human breast tissue was obtained from females having plastic surgical operations. Placenta tissue from healthy mothers was collected within 4 hr after fetal birth.

DNA preparation. The DNA was extracted either by the method of Marmur[12] or according to Kirby and Cook[13]. Additional purifi-

cation was often carried out by adsorption of the DNA to hydroxyapatite and subsequent washing with 0.2 M sodium phosphate dissolved in 3 M KCl. In some cases, the DNA was treated with 100 μg/ml alpha-amylase and boiled ribonuclease A for 18 hr at room temperature. Digested products were removed by dialysis against 0.1xSSC (SSC=0.15 M NaCl + 0.015 M sodium citrate). The DNA was further treated with 1 mg/ml self-digested pronase for 2 hr at 37°C. The resultant solutions were next adjusted to 1 M NaCl and cleaned with phenol-m-cresol[14] until they were below 0.08 μg protein/μg DNA as determined by the method of Lowry[15]. However, difficulty in removing the added enzymes from the DNA was encountered, and so this procedure was abandoned in later experiments.

When sheared DNA was desired, the solution was made 2xSSC and 10-15 ml were placed into a 50 ml plastic centrifuge tube already immersed in an ice bath. The microtip of a Branson sonifier (model S125) was lowered into the DNA solution and 1-10 10-sec pulses with 1 min intervals under a power supply setting of 6 were carried out.

In some cases the DNA was centrifuged to equilibrium in CsCl at an initial density of 1.710 g/cm^3. Centrifugation was at 44,000 rpm for 72 hr at 15°C. The Ti 50 rotor was used with 200-300 μg DNA/gradient and 6-8 drop fractions were collected. The Ti 60 rotor was used with 750-1000 μg DNA/gradient and 12-15 drop fractions were collected.

DNA determination. In order to determine quantitatively the per cent hybridization of (^{3}H)poly(U) to DNA, it was essential to know precisely the amount of DNA that remained after the hybridization procedure. For this purpose the diphenylamine method of Richards[16] was adapted. Nitrocellulose filters containing DNA that had been hybridized to (^{3}H)poly(U), RNAase treated, and the radioactivity counted were washed free of scintillation fluid with chloroform. The dried filters were then each incubated in 1.2 ml of 1.6 M perchloric acid at 70°C for 30 min. After heating, 0.7 ml of the diphenylamine reagent (4 % diphenylamine + 0.01 % paraldehyde in glacial acetic acid) was mixed with the perchloric acid and the filters removed. The resulting solution was incubated for 18 hr at 30°C and the absorbance at 600 nm recorded. The relationship of absorbance to DNA concentration on the filters was linear for salmon sperm DNA and (^{3}H) thymidine labelled

human DNA from 1-20 μg. The minimal detection limit was considered to be 1 μg DNA/filter which was equal to 0.020 optical density units at 600 nm.

(^{3}H)poly(U)-DNA filter hybridization technique. The DNA was denatured with heat for 10 min at 100°C. An equal volume of 4 M NaCl was added and the heat continued for another 10 min. The solutions were cooled immediately and the DNA immobilized at 5°C on 13-mm Millipore HAWP 01300 filters presoaked in 2xSSC for at least 30 min. The high salt treatment of the DNA increased retention to the filters, and was also a precaution for removing any histone-type protein that may still be bound to the DNA. Hybridization vials (1.5 cm x 5.0 cm) were constructed to allow continuous circulation of the (^{3}H)poly(U) solution with the aid of a peristaltic pump. The filters containing immobilized DNA were hybridized in bulk for 16-20 hr at 25°C in (^{3}H)poly(U) dissolved in 2xSSC. Optimal temperature for hybrid formation in 2xSSC solution have been determined to be 20°C to 30°C for eukaryotic DNA[7,9]. Based on these results, 25°C was accepted as the hybridization temperature. The specific activity of the (^{3}H)poly(U) sources was diluted to 30,000-50,000 cpm/μg with non-radioactive poly(U) to provide a larger supply of labelled material. The poly(U) solutions could be stored for 1-2 months at -20°C before their ability to hybridize DNA was reduced. Some (^{3}H)poly(U) sources arrived at our laboratory with an inferior capacity to hybridize DNA on filters, and therefore each new source should be tested before use. Following incubation, the (^{3}H)poly(U) solution was decanted off and the filters incubated for either 30 min or 6 hr at 25°C with pancreatic ribonuclease A dissolved in 2xSSC. The filters were then washed 3 times in 2xSSC, oven-dried and counted in 10 ml toluene-based scintillator fluid (0.5 % PPO and 0.03 % POPOP in toluene). The efficiency of (^{3}H) counting was 44 % on nitrocellulose filters. The % (dA) regions in the DNA were calculated by dividing the μg (^{3}H)poly(U)/μg DNA x 100 by 2. All hybridizations in this study were performed on filters as just described, unless specifically stated otherwise.

Hydroxyapatite fractionation of DNA. Sheared DNA was dialyzed against 0.12 M phosphate buffer composed of equimolar amounts of Na_2HPo_4 and NaH_2Po_4, pH 6.8. The DNA was then dena-

tured for 15 min at 100°C and placed immediately in a water bath at 60°C for reassociation. The reassociation was measured as the product of DNA concentration (C_o) and the time (t) of incubation or the $C_o t$ value ($C_o t = A_{260} \times \frac{n}{2}$). After reassociation at specified $C_o t$ values, the DNA was loaded onto a glass column of hydroxyapatite equilibrated in 0.12 M phosphate buffer at 60°C, and containing at least 1 ml hydroxyapatite for every 200 µg DNA. Unreassociated DNA was eluted in 0.12 M phosphate buffer. The reassociated DNA was removed by raising the buffer concentration to 0.4 M phosphate.

RESULTS

Determination of the conditions for poly(U) hybridization on filters. One basic problem in adapting the filter hybridization technique to the estimation of (dA) regions in DNA, is to determine whether (dA) sequences can be retained quantitatively in an immobilized state on nitrocellulose filters. For this purpose, pure poly(dA) was dissolved in various salt solutions and loaded onto nitrocellulose filters. Table 1 indicates that the salt concentration must be very high (2 M NaCl) before poly(dA) is retained quantitatively on the filters. Furthermore, when the poly(dA) is fixed to the filters by heating for 2 hr at 80°C, no substantial loss of poly(dA) is observed after exposure of the filters to the poly(U) hybridization conditions outlined

TABLE 1

The collection of pure poly(dA) on nitrocellulose filters.

Attempted immobilization of poly(dA) (Sigma) was with 15 µg/sample on nitrocellulose filters (millipore). The amount of poly(dA) retained on the filters was measured by the diphenylamine procedure described in Materials and Methods.

Treatment of poly(dA)	% Retention
Loaded on filters in 2xSSC	0
Loaded on filters in 6xSSC	11
Loaded on filters in 2 M NaCl	77
Loaded on filters in 2 M NaCl Baked 2 hr at 80°C Incubated for 18 hr in 2xSSC at 25°C Washed 3 times in 2xSSC	80

in Materials and Methods. A 75 % to 80 % (dA) retention was considered quantitative, since immobilization of denatured human DNA was in the range of 75 % - 95 % retention. This somewhat low retention was probably due to the fact that our DNA samples were often alcohol precipitated and the precipitate collected by centrifugation only. These results contradict those found by Shenkin and Burdon[8]. However, although not stated explicitly, their high salt immobilization medium was probably the same as their hybridization solution (2xSSC). If this was true, then even according to our results poly(dA) would not be retained.

It has been shown[8] that increasing the concentration of ribonuclease A, or the duration and/or the temperature of incubation can cause large reductions in the amount of poly(U) remaining bound to the DNA following hybrid formation in solution. Mild ribonuclease treatment (5 μg/ml at 20°C for 30 min) allowed larger (dA) rich regions, containing 2 % to 6 % other deoxynucleotides, to form ribonuclease resistant complexes with poly(U). Exhaustive digestion (20 μg/ml ribonuclease at 20°C for 24 hr) allowed only the poly(U) complexes with the shorter (dA) pure regions of the DNA to remain stable. Based on the importance of these results in quantitating the poly(U)-DNA hybridization, the conditions for appropriate ribonuclease treatment of hybrids between poly(U) and immobilized pure poly(dA) were determined.

Fig. 1 confirms the earlier work of Bishop et al[9] that the ribonuclease-resistant complex formed between poly(U) and poly(dA) is the triplex 2 poly(U)-poly(dA), since the experimental hybrid ratio of approximately 1.8 : 1 is close to the hypothetical ratio of a 2 : 1 complex. It is also apparent that the 2 poly(U)-poly(dA) triplex is resistant to digestion at 25°C in 2xSSC for 7 hr at 5 μg/ml ribonuclease but only for 1 hr at 20 μg/ml ribonuclease. After these points on the digestion curves, the (dA) stretches in DNA could no longer be estimated quantitatively as 2 poly(U)-poly(dA) triplexes. Therefore, in keeping with the nomenclature established by Shenkin and Burdon[8], the larger more mismatched (dA) rich regions in human DNA will henceforth be estimated after mild ribonuclease treatment (5 μg/ml at 25°C for 30 min) and the smaller more (dA) pure regions estimated after exhaustive ribonuclease treatment

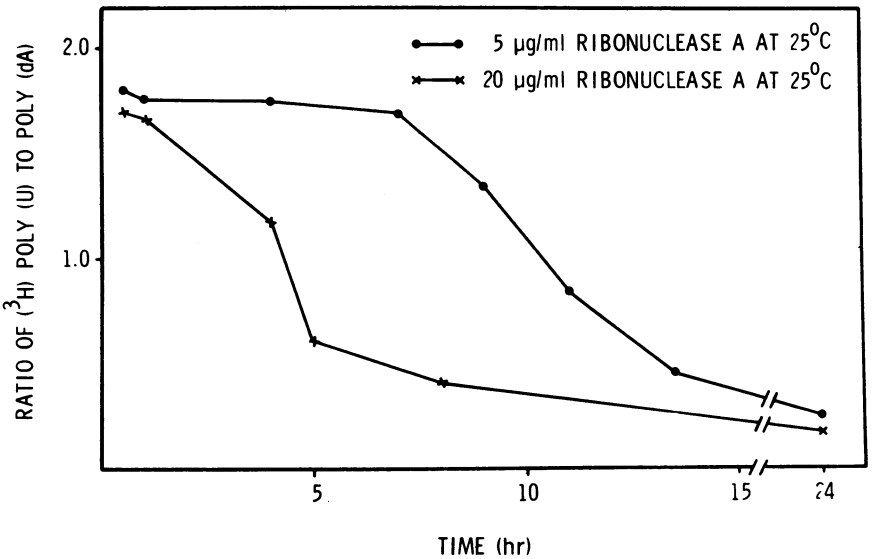

FIG.1 Stoichiometry of reactions between poly(U) and poly(dA) and the ribonuclease-resistance of the resulting hybridized complexes.

Pure poly(dA) (Sigma) was immobilized on nitrocellulose filters (0.2 µg/filter) in 2 M NaCl. Each filter was baked in an oven at 80°C for 2 hr and then hybridized at a (^{3}H)poly(U) to poly(dA) input ratio of 5.69 : 1.

(5 µg/ml at 25°C for 6 hr).

The saturating level of (^{3}H)poly(U) input was also determined for hybrids formed on filters. As can be seen in Fig. 2, it was necessary to incubate the filters in 4.2 µg/ml (^{3}H)poly(U) before an increase in (^{3}H)poly(U) concentration did not result in a corresponding increase in the % hybridization of poly(U) to DNA. Without any ribonuclease treatment approximately 0.028 µg poly(U) binds to 1 µg human DNA. Such a large amount of poly(U) binding to DNA can alter the saturation threshold by reducing the concentration of poly(U) that is available in the hybridization solution to drive the annealing reaction to completion. Therefore, to insure saturation in a filter hybridization with poly(U), at least 3.6 µg/ml poly(U) must remain after hybrid formation is completed. This can be calculated by substracting the expected amount of poly(U) bound to DNA before ribonuclease treatment (o.028 x 20 µg DNA, from Fig. 2) from the concentration of poly(U) necessary to achieve saturation (4.2 µg/ml).

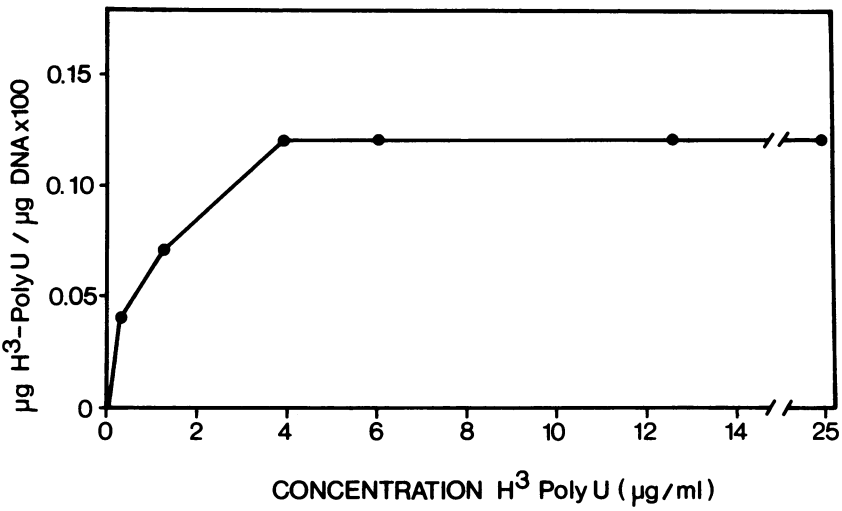

FIG.2 The saturation curve for hybridization of poly(U) to human DNA on nitrocellulose filters.

Immobilized human DNA (20 µg) was incubated in 1 ml of increasing concentrations of (^{3}H)poly(U) and the hybrids analyzed as in Materials and Methods. Ribonuclease treatment was 5 µg/ml at 25°C for 6 hr.

Level of (dA) regions in human DNA. The (dA) regions in human DNA were estimated by using both filter and liquid hybridization techniques to poly(U) followed by comparable ribonuclease treatments. When the (dA) regions were measured after mild ribo-

TABLE 2

The level of (dA) rich and (dA) pure sequences in human DNA.

Liquid hybridization was with 10 µg heat-denatured DNA and 1 µg (^{3}H)poly(U) dissolved in 1 ml 2xSSC for 4 hr incubation at 25°C. Ribonuclease treatment as described in the text for estimation of (dA) rich and (dA) pure sequences was carried out directly in the hybridization mixtures. The ribonuclease-resistant hybrids were collected on nitrocellulose filters by 7 % TCA precipitation.

Human DNA	Hybridizing conditions	% (dA) rich sequences (RNAase 30 min)	% (dA) pure sequences (RNAase 6 hr)
Breast	Filters	0.39	0.070
Lymphocyte	Filters	0.38	0.075
Placenta	Filters	0.37	0.068
Lymphocyte	Liquid	0.40	0.074

nuclease digestion, 0.39 % of the DNA contained (dA) rich sequences (Table 2). The more (dA) pure regions, which were estimated after exhaustive ribonuclease digestion, were 0.07 % of the DNA. No differences were observed between the values calculated by either the filter or liquid hybridization techniques. The fact that human DNA extracted from 3 different sources gave essentially the same values for the (dA) levels indicates the reproducibility of using the filter technique with poly(U)-DNA hybridizations.

Two factors were shown to effect the analysis of (dA) regions in human DNA. They were the level of protein contamination of the DNA and the degree to which the DNA was sheared. Table 3 shows that 18.1 % protein contamination of DNA was necessary before the estimation of (dA) pure sequences was altered. In our laboratory, such a high level of protein contamination was not normally found with DNA extracted by conventional methods[12,13]. However, when additional purification of DNA was attempted by addition of the digestive enzymes alpha-amylase, ribonuclease A, and pronase, high levels of protein contamination of the DNA were often obtained even after several phenol deproteination steps. Therefore, care should be taken to check the protein content of DNA which has been further purified by digestive enzyme treatment.

TABLE 3

The effect of protein contamination of human DNA on the analysis of (dA) regions.

Protein was analyzed by the method of Lowry[15]. Bovine serum albumin and highly purified DNA were used as standards.

Protein content of DNA µg protein/µg DNA x100	(dA) rich sequences (RNAase 30 min)	(dA) pure sequences (RNAase 6 hr)
2.0	0.38	0.072
8.0	0.39	0.070
18.1	0.35	0.053[a]

[a] $p \leq 0.05$ when compared to less protein contaminated DNA

Shearing DNA by 100 sec sonication as already described resulted in molecular weights averaging 4.4×10^5 as determined

in 5-20 % neutral sucrose gradients according to the relationship derived by Studier[17]. A significant effect on the estimation of (dA) pure regions within sheared DNA of this molecular weight range was detected in the data presented in Table 4. Therefore, comparison of very low molecular weight DNA with high molecular weight DNA is not recommended when using the filter hybridization technique.

Distribution of (dA) regions in human DNA. Previously it has been reported for hamster[8], mouse[7] and duck[9] DNAs that the (dA) regions were rather evenly distributed into the fractions of DNA generated from centrifugation in CsCl gradients. Fig. 3 shows the results of a poly(U) filter hybridization to CsCl gradient fractions of human DNA, when the actual % poly(U) hybridizing to each DNA fraction was determined. Although (dA) regions could be found in all the DNA fractions, our results indicated a lower level of (dA) regions in the more G+C rich and A+T rich DNA fractions. These results were confirmed by

TABLE 4

The effect of shearing on the analysis of (dA) regions in human DNA immobilized on nitrocellulose filters.

Sonication time of sheared DNA (sec)	(dA) rich sequences (RNAase 30 min)	(dA) pure sequences (RNAase 6 hr)
0	0.37	0.073
10	0.39	0.067
20	0.36	0.075
30	0.38	0.072
40	0.36	0.068
50	0.43	0.070
100	0.41	0.052[a]

[a] $p \leq 0.05$ when compared to less sheared DNA

pooling the DNA fractionated in CsCl gradients into G+C rich, main band and A+T rich fractions, and then hybridizing the pooled fractions to poly(U). The data in Table 5 demonstrate that main band DNA clearly has a higher level of both (dA) rich and (dA) pure sequences than either the G+C rich or A+T rich fractions.

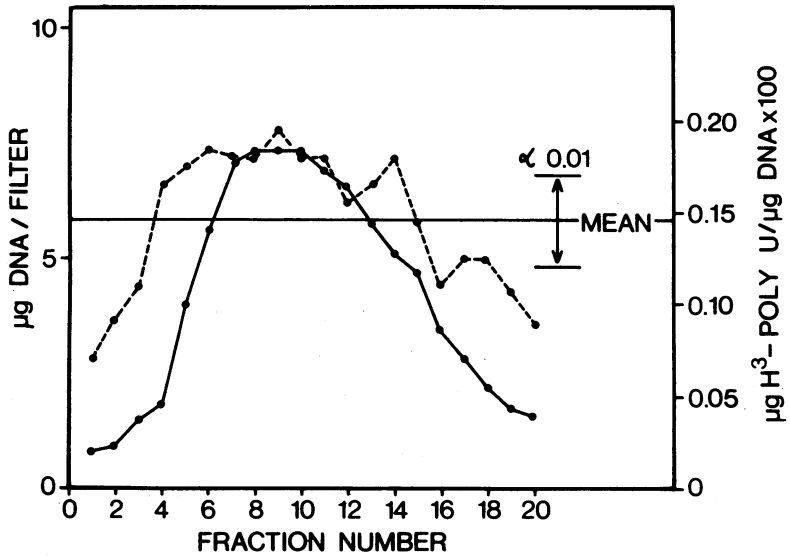

FIG.3 The per cent hybridization of poly(U) to CsCl gradient fractions of human DNA.

The CsCl gradients were prepared, fractionated and the DNA analyzed by the filter hybridization technique described in Materials and Methods. Ribonuclease treatment was 5 µg/ml at 25°C for 6 hr. The density increases from right to left. DNA concentration (●——●); poly(U) hybridization (●- - -●).

TABLE 5

Comparison of (dA) regions in the pooled CsCl density gradient fractions of DNA from Fig. 3.

The CsCl fractions were pooled according to the fraction numbers indicated in Fig. 3 and dialyzed overnight against 2xSSC. Aliquots of the pooled fractions were immobilized on filters and hybridized as described in Materials and Methods.

Pooled DNA fractions	(dA) rich sequences (RNAase 30 min)	(dA) pure sequences (RNAase 6 hr)
1- 7 (G+C rich)	0.35	0.065
8-10 (main band)	0.52	0.097
11-20 (A+T rich)	0.31	0.056

About 35 % of human DNA are families of repeated sequences[18]. The heterogenous nature of repetitive DNA in the human genome has been well established by thermal elution chromato-

graphy on hydroxyapatite[18]. Virtually every temperature elution contained both fast and slow reassociating fragments of both A+T and G+C rich base composition. These facts suggested that at least some repeated sequences in the DNA would contain (dA) regions. The data presented in Table 6 has verified the presence of (dA) regions in repeated human DNA. Higher levels of both (dA) rich and (dA) pure sequences were found in all the different classes of repeated DNA analyzed when compared to single copy DNA. These increased (dA) levels correlated well to the degree of repetition, so that fast repeated DNA had the largest increased (dA) level and slow repeated DNA the least (Table 6).

TABLE 6

Distribution of (dA) regions into repeated sequences of human DNA.

The DNA was sheared by sonication for 100 sec, fractionated by hydroxyapatite chromatography into repeated classes, and hybridized as described in Materials and Methods.

Reassociated DNA fraction	% native DNA	(dA) rich sequences (RNAase 30min)	(dA) pure sequences (RNAase 6hr)
C_0t 0.0 - 0.05 Fast repeated	23.7	0.65[a]	0.084[a]
C_0t 0.05 - 1 Intermediate repeated	8.4	0.35[a]	0.037[a]
C_0t 1 - 50 Slow repeated	9.9	0.26[a]	0.027[a]
C_0t 50 - Single copy	58.0	0.16	0.018

[a] $p \leq 0.05$ when compared to single copy DNA

DISCUSSION

Estimates of the % poly(U) hybridizing to mammalian DNA have ranged from 0.12 % to 0.55 %[7-10]. Our calculation of 0.78 % poly(U) complexing with human DNA after similar ribonuclease

treatment was considerably higher (Table 2, triplex = 0.39 x 2). One possible explanation for this discrepancy may be that the amount of DNA hybridized to poly(U) was not quantitatively determined in the previous studies. Quantitative retention of DNA approaching 100 % was apparently assumed, since no techniques for DNA determination were reported[7-9]. If, in fact, DNA retention was less than 100 % then the poly(U) hybridization values would have been actually larger than those reported.

Birnboim et al[19] have determined by formic acid-diphenylamine analysis that approximately 0.5 % of human DNA is arranged into (dT) rich sequences. However, no absolutely pure (dT) stretches were found ($<$0.0015 % of native DNA). Presumably the same would be true for complementary absolutely pure (dA) regions in the DNA. The estimate of $<$ 0.0015 % pure (dT) regions is much lower than the 0.07 % (dA) pure regions in human DNA calculated from our results (Table 2). Such a wide variation suggests that the absolutely pure (dA) regions in DNA (if they exist at all) could not be estimated as a 2 poly(U)-DNA triplex, since (dA) stretches which are relatively pure but contain a few other deoxynucleotides form ribonuclease-resistant triplexes as stable as does pure (dA) itself.

Our results (Fig. 3, Table 5) suggest that main band DNA is enriched for (dA) sequences. Recently, Tapiero et al[20] have reported that the main band DNA in chick has a higher proportion of single copy and slow repeated DNA than does G+C rich or A+T rich DNA. Since most mRNA is transcribed from single copy template in the DNA[21-26], there may be a correlation between potential mRNA transcription sites and the distribution of (dA) sequences associated with the single copy DNA. Moreover, (dA) sequences are also concentrated into highly repetitive DNA (Table 6). It has been reported that HnRNA or precursor mRNA have oligo(U)[4,5] and oligo(A)[6] units present in their nucleotide sequences which are transcribed from repetitive DNA template[5]. The oligo(U) and oligo(A) units are found only in large molecular weight HnRNA and not in the lower molecular weight cytoplasmic mRNA[5,6]. These observations support a possible involvement of (dA) regions in DNA as important sites of transcription for processing of genetic information.

ACKNOWLEDGEMENTS

The present work has been supported by grants from the Swedish Natural Sciences Research Council, the Swedish Cancer Society and the John and Augusta Persson Foundation.

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