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A Limited Number of Globin Genes in Human DNA

(complementary DNA/thalassemia)

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ABSTRACT **The number of globin genes in human cells was determined by hybridizing DNA from human spleens to ^{3}H-labeled DNA complementary to human globin mRNA. Assuming the rates of reannealing of complementary DNA and cellular DNA are similar, the extent of hybridization of complementary DNA at various ratios of cellular DNA to complementary DNA indicate that there are fewer than 10 globin gene copies per haploid human genome. An alternative analysis of the data, which introduces no assumptions concerning the relative rates of reaction of complementary DNA and cellular DNA, indicates fewer than 20 globin gene copies are present. DNA isolated from the spleen of a patient with β^+ thalassemia contained a number of globin gene copies similar to that of normal DNA.**

The mechanisms regulating human hemoglobin synthesis at the gene level are poorly understood. Since both transcriptional and translational controls may be involved, it is of importance to determine the number of globin genes in man. The availability of highly radioactive DNA (cDNA) complementary to globin mRNA (1–3) provides a useful tool for this purpose. Recent reports of the use of mouse liver and duck reticulocytes indicate that there are fewer than 10 copies of the globin sequences present per haploid genome (4–6). In addition, the number of globin genes present in duck reticulocytes and liver cells has been found to be similar (4). The experiments reported here were undertaken to measure the number of globin genes present in human genomes, and to compare the number of genes in the cells of patients with β^+ thalassemia and without thalassemia. Human cDNA was used to probe DNA from human spleens for the number of globin gene copies. The results are consistent with genetic evidence, and indicate that fewer than 20 globin genes are present in normal haploid human genomes. In addition, there is no detectable difference in the number of globin genes present in DNA from the cells of a patient with β^+ thalassemia.

METHODS

Preparation and Characterization of Human DNA. Human DNA was isolated from individual spleens of patients with and without thalassemia obtained when splenectomy was indicated for treatment of the patient. The spleens were collected within 1 hr after surgery, cut into small pieces, frozen immediately in liquid nitrogen, and stored at −70°. The spleens were ground to a coarse powder in a mortar cooled with dry ice, and the DNA was isolated by the following procedure modified from those reported (7, 8): Frozen spleen powder (10–50 g) was suspended at 4° in 5% sucrose buffer containing 1 mM $MgCl_2$ and 1 mM NaH_2PO_4 (pH 6.5). The cells were broken by 25 strokes in a tight-fitting Dounce homogenizer. The nuclei were separated by centrifugation at 800 × *g* for 10 min, washed once with the 5% sucrose solution, and taken up in 10–20 volumes of 10 mM Tris (pH 8.3), 0.15 M NaCl, 5 mM EDTA, 1% sodium dodecyl sulfate, 1.0 M $NaClO_4$. After addition of an equal volume of $CHCl_3$–isoamyl alcohol (24:1), the mixture was shaken for 30 min at room temperature. The aqueous phase was removed and the material at the interphase was again extracted. The pooled aqueous phases were extracted until no significant precipitate remained at the interphase. Two volumes of 95% ethanol were layered over the aqueous solution and the DNA was collected on a glass rod. The DNA was dried under reduced pressure and dissolved in 1.5 mM NaCl, 0.15 mM sodium citrate. The DNA solution (0.5–1 mg/ml) was treated for 2 hr at 37° with RNase A (50 μg/ml, Sigma, Type 3A) and, under similar conditions with Pronase (50 μg/ml, Calbiochem, nuclease-free B grade). Sodium dodecyl sulfate was added to 1%, and the DNA was extracted twice with phenol–cresol–hydroxyquinoline solution (8). The DNA was again collected under ethanol and dissolved in 1.5 mM NaCl, 0.15 mM EDTA (pH 7.0) at 1 mg/ml; NaOH was added to 0.01 M, and the DNA was sheared by sonication. The DNA was then extracted with phenol–cresol solution, precipitated with ethanol, dried, and dissolved in 0.12 M phosphate buffer (pH 6.8), 1 mM EDTA.

Isolation of Globin mRNA. Human globin mRNA was prepared from reticulocytes by phenol extraction and subsequent oligodeoxythymidylate [oligo(dT)] column chromatography, as described (9, 10). The mRNA used was biologically active when added to a Krebs ascites tumor cell-free system (10, 11).

Human DNA·[^{3}H]cDNA Hybridization. Globin cDNA labeled with [^{3}H]dCTP (26 Ci/mmol, ICN) was prepared as described (1). Hybridizations were performed in 50 μl of 0.12 M sodium phosphate (pH 6.8), 0.4% dodecyl sulfate. Reaction mixtures were assembled in 100-μl capillary pipettes, heated in a boiling H_2O bath for 15 min, and incubated at 68°. At various times, the capillaries were frozen on dry ice. For analysis, the contents were expelled into 2 ml of 0.12 M sodium phosphate (pH 6.8), 0.4% dodecyl sulfate and applied to 1-g columns of hydroxylapatite (Bio-Rad) equilibrated with the same buffer at 68°. The nucleic acids were eluted with 3 aliquots of 2 ml each of the same buffer and subsequently

Abbreviations: cDNA, DNA complementary to human globin mRNA; C_0t, product of concentration of DNA nucleotides and time of incubation; T_e, melting temperature of hybrids measured as the midpoint of the elution profile from hydroxylapatite.

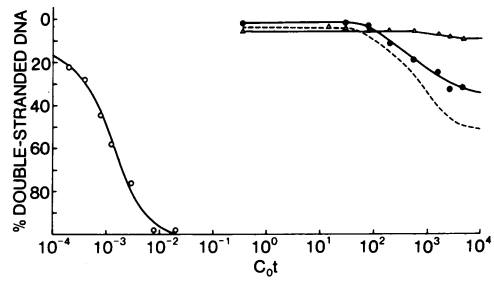

FIG. 1. Kinetics of hybridization of 10S globin RNA and DNA from various species to cDNA. cDNA (0.96 μg) was hybridized to 1.8 mg of DNA for various times to obtain C_0t values. The percentage of double-stranded DNA was determined by hydroxylapatite chromatography (see *Materials and Methods*). ●, Human 10S RNA; △, *E. coli* DNA; ●, calf thymus DNA; broken line, human normal DNA. The points of the latter hybridization are in Fig. 2.

with 0.4 M sodium phosphate (pH 6.8), 0.4% dodecyl sulfate. Under these conditions, single-stranded DNA elutes in 0.12 M phosphate and double-stranded DNA in 0.4 M phosphate (12).

RESULTS

Characterization of cDNA and Cellular DNA. The extent of homology between the cDNA and its template was determined by the use of micrococcal nuclease (13). The cDNA hybridizes completely to the globin mRNA and contains no noncomplementary "tails" (Fig. 1). The hybrids formed are stable and exhibit a sharp melting curve when eluted from hydroxylapatite by increasing temperature (10). The T_e (temperature of the hybrids at the midpoint of the elution profile, analogous to the T_m measured in solution) is 90°. This value is the same as that found for rabbit globin mRNA·cDNA hybrids (14) and indicates a high fidelity of transcription of the RNA sequences into DNA.

The sizes of the sonicated cellular DNA and the cDNA probe were determined by sedimentation in alkaline sucrose gradients, with φX174 DNA (18 S) as reference. The cDNA sedimented at about 7 S, indicating a molecular weight of about 200,000. This value is identical to that obtained with rabbit cDNA. The cellular DNA gave a broader profile, somewhat larger in size. The average molecular weight was 280,000, or about 1.4 times larger than the cDNA.

Determination of the Number of Human Globin Genes. The number of genes coding for globin in man was determined by hybridizing in solution various amounts of globin cDNA to human cellular DNA. Under these conditions the cDNA competes for its genome complement with the other strand of cellular DNA. The rate and extent of the hybridization reaction will thus depend upon the ratio of cDNA to cellular DNA, as well as the rates of the two competing reactions. If we assume that these two rates are the same, the analysis becomes straightforward.

We designate the cDNA as (+) and those sequences complenentary to it as (−). The cellular DNA contains equal numbers of its own (+) and (−) globin sequences; therefore, by adding [^{3}H]cDNA we create an excess of (+) strands over the complementary (−) strands. Hybridization can only proceed until the (−) strands are exhausted, and the reaction will terminate before all the cDNA has hybridized. The final extent of hybridization thus depends upon the initial ratio of cDNA to cellular DNA. The percentage, P, of cDNA taken up into hybrids is equal to the fraction of total (+) strand hybridized, since the labeled and unlabeled material are assumed to behave the same. Since the amount of total (+) strand hybridized is equal to the amount of cellular (−) strand sequences, we may write:

$$P = \frac{(+)\text{ strand hybridized} \times 100}{\text{total }(+)\text{ strand}} = \frac{\text{ng of }(-)\text{ cellular globin DNA} \times 100}{\text{ng of }(+)\text{ cellular globin DNA} + \text{ng of }(+)\text{ cDNA}} \quad [1]$$

Since the cellular (+) and (−) sequences are present in equal amounts and the quantity of cDNA is known, we can determine P and solve for the amount of globin sequences present in the cellular DNA used in the hybridization reaction (Table 1).

TABLE 1. *The number of globin sequences present in human cellular DNA*

Expt.	Input cDNA (ng)	% Hybridization at saturation* Expected for no. of copies 1	5	10	Observed†	No. of gene copies
Nonthalassemia						
1	0.32	38	75	86	59	2–3
2	0.96	17	51	67	47	5
3	3.20	6	24	38	30	7
Thalassemia						
1	0.32	38	75	86	59	2–3
2	3.20	6	23	38	30	7

* The specific activity of the cDNA is 1.4×10^7 cpm/μg (assuming 80 dCTP molecules per cDNA). 4500 cpm were used in reaction 1, representing 0.32 ng of input cDNA. The human haploid genome is 1.8×10^{12} daltons (27). The globin gene is about 2×10^5 daltons. The fraction of the total human genome that is globin gene is, therefore, 1.1×10^{-7}. Cellular DNA (1.8 mg) was used in each hybridization reaction. If a single globin gene were present, the input DNA (1.8 mg) would contain $1.8 \text{ mg} \times 1.1 \times 10^{-7}$, or 0.198 ng of globin DNA. From Eq. [1] we can calculate the % hybridization, P, expected for any number of globin gene copies present. For example, in Exp. 1, if one globin gene is present, there would be 0.198 ng of cellular globin DNA and $P = (0.198 \times 100)/(0.198 \times 0.32) = 38\%$. If five globin genes are present, we would expect 5×0.198 ng of globin gene DNA in the cellular DNA and $P = (5 \times 0.198 \text{ ng})/(5 \times 0.198 \text{ ng} + 0.32) = 75\%$. Since we observed $P = 59\%$ hybridization, there are between 1 and 5 cellular globin gene copies by this calculation. The expected percent saturations for different numbers of globin gene copies have been calculated for various cDNA inputs by this method.

† Background (4%) has been subtracted.

We have performed hybridizations at three different ratios of cDNA to human spleen cellular DNA. The results obtained are shown in Fig. 2 and Table 1 (15). An analysis of the results by the method described above indicates that there are fewer than 10 copies of the globin genes per haploid human genome (Table 1). The percent hybridization at saturation is reproducible in duplicate experiments within 2–5%. The lack of hybridization of cDNA to cellular DNA at a C_0t less than 10 indicates that the probe is free of contaminating ribosomal RNA complements and will not hybridize to repetitive DNA species. The T_e of the hybrids formed between cellular DNA

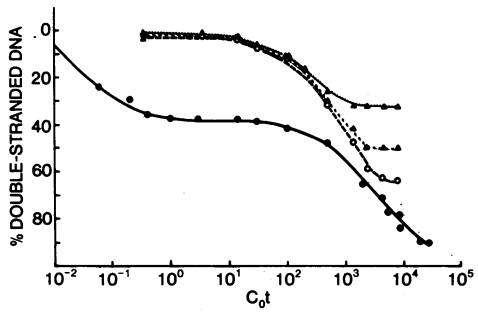

FIG. 2. Hybridization of normal cellular DNA with cDNA. Each reaction contained 1.8 mg of cellular DNA. The time was varied to obtain the C_0t values shown. Percent of double-stranded DNA was measured by hydroxylapatite chromatography. The amount of cDNA was varied: O, 0.32 ng of cDNA; ▲, 0.96 ng of cDNA; △, 3.2 ng of cDNA; ●, The % cellular double-stranded DNA (as A_{260}).

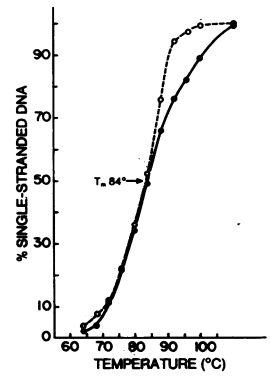

FIG. 3. Melting profile of cellular DNA and cDNA·DNA hybrids. Hybridization of cDNA to cellular DNA and thermal elution of single-stranded DNA was performed as described in *Materials and Methods*. ●, Cellular DNA (A_{260}); O, cDNA·DNA.

and cDNA is 84°, indicating that the vast majority of the DNA·cDNA hybrids are faithfully base-paired (Fig. 3). This T_e is identical with that of the renaturing cellular DNA (Fig. 3). No significant hybridization was observed between the cDNA probe and *Escherichia coli* DNA. The extent of hybridization with calf thymus DNA was less than in the homologous system, as expected (Fig. 1).

The progressive increase in copy number as the ratio of cDNA to cellular DNA increases suggests that the assumption that the reannealing cDNA and cellular (+) globin DNA to (−) cellular globin DNA occurs at the same rate, is not completely correct. Treatment of the data without this assumption is presented in the *Appendix*. The results of this latter analysis indicate that fewer than 20 copies of globin genes are present in the haploid human genome, and that the rate of hybridization of cDNA is only $^1/_3$ that of reannealing cellular globin DNA sequences.

Hybridization of [^{3}H]cDNA with β Thalassemia Cellular DNA. DNA was isolated from a patient with β^+ thalassemia and hybridized to cDNA. The results at two concentrations of cDNA are comparable to those obtained with the same amounts of cellular DNA from nonthalassemic subjects (Table 1). In another experiment, rabbit mRNA enriched 4- to 5-fold for β globin mRNA was used to prepare [^{3}H]cDNA, which was hybridized to spleen DNA from a patient without thalassemia and to that from the β^+ thalassemic subject. The kinetics and extent of hybridization were the same with both samples. About 32% hybridization occurred at saturation, compared with 59% hybridization obtained with an identical amount of homologous probe. The lower level of hybridization with the enriched β globin rabbit cDNA probe than with human cDNA is probably due to the noncomplementary regions between rabbit cDNA and human DNA (16).

DISCUSSION

The results of these studies indicate that there are fewer than 20 globin genes present in haploid human genomes. There are six types of globin genes in human genomes: α, β, δ, γ, ε, and θ (17). The cDNA probe was prepared with globin mRNA from adult reticulocytes synthesizing predominantly α and β globin chains; thus, faithful hybridization of the cDNA would measure only α and β globin genes. The δ chains, however, differ from β chains by only nine amino acids, and β cDNA might be expected to crossreact with δ DNA. It is less likely that γ genes will crossreact since there are differences in 38 amino acids between β and γ chains, but even a small region of homology is sufficient to detect the gene by hydroxylapatite chromatography. It is unknown whether the amino-acid homologies are faithfully reflected at the nucleotide level. We can only speculate on the relative number of α, β, γ, δ, and other genes represented in our studies. At one extreme, we would suggest that the minimum of three genes measured includes two α globin genes and one β globin gene. These values agree with genetic studies of several types (17). It is, however, possible that δ, γ, and other globin genes are also being measured. The precise number of each of these genes is unknown in humans; however, at least two, and perhaps more, γ genes are present in haploid human genomes (18). In addition, untranscribed globin genes may be present; for example, so-called minor, multiple δ globin genes have been reported in apes (19). The small number of globin genes present in the human genome are in agreement with the findings in studies of nonerythroid cells and erythroid cells at different stages of development in other species (20). These results suggest that the large amounts of globin mRNA present in erythroid cells are not due to a large number of globin genes, but rather to an increase in globin gene transcription.

In the erythroid cells of patients with β thalassemia, there is a decreased amount of β mRNA present, as determined by biologic activity assay in cell-free systems (21–23) and by molecular hybridization (16, 24). The underlying gene defect responsible for reduced β globin mRNA synthesis may be due to (*1*) deletion of β globin genes, (*2*) a regulatory gene mutation with repression of β globin genes, or (*3*) abnormal processing of β globin mRNA from heterogeneous nuclear RNA. This first possibility would theoretically occur only in so-called β° thalassemia, since genetic data indicate that only one β globin gene is present per haploid chromosome set. Since the human β gene is probably unique (17), and represents only a small fraction of the total globin genes present, it is not surprising that we observed no difference in the total number of globin genes between the genomes of normal subjects and of one with β^+ thalassemia. Hybridizations with other β^+, as well as β°, thalassemia patients will be necessary to evaluate whether deletions of the β globin gene sequences can account for any of the β thalassemia syndromes. However, we can conclude that there is no extensive deletion of globin genes in the one β^+ thalassemia patient studied. The isolation of cDNAs specific for α, β, δ, and γ mRNAs will be necessary to quanti-

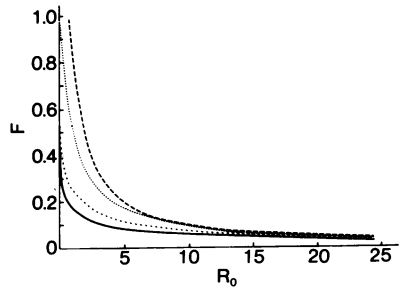

FIG. 4. Relationship of ratio of amount of cDNA and cellular RNA (R_0) to fraction of cDNA hybridized (F), by Eq. [2] in *Appendix*. The k_1/k_2 values used were as follows; (---) 0.1; (···) 1.0; (-·-) 5.0 ; and (—) 10.0.

tate more precisely the number of each of the globin genes in the genomes of patients with and without thalassemia.

Studies of globin gene regulation would be greatly aided if globin genes containing operator sequences could be obtained in pure form. The specificity of hybridization of cDNA to cellular globin gene sequences is potentially of use in experiments whose aim is the isolation of human globin genes.

APPENDIX

Using a simplified method of calculating the number of globin genes, we found a progressive increase in the copy number as the ratio of cDNA to cellular DNA was increased. This result suggested that the rate of hybridization of cDNA with its cellular complement might not be the same as the rate of the reannealing of the two cellular globin DNA strands. We have, therefore, chosen to analyze our data by procedures that make no assumptions regarding the rates of the two competing reactions.

Straus and Bonner (25) have derived the equations necessary to describe the system under study. In particular, they show that

$$\frac{k_2}{k_1} = \frac{\log (1-F)}{\log R_0 + \log F} \qquad [2]$$

where k_2 is the rate constant for the hybridization of the cDNA with its complement; k_1 is the rate constant for the reannealing of complementary cellular globin DNA sequences; R_0 is the ratio of the concentration of probe to that of its complementary cellular DNA sequences; and F is the final extent of hybridization expressed as a decimal fraction.

Those authors applied the equation to systems in which the DNA sequences were in moderate excess over an RNA probe. The derivation of the equations they used did not, however, make any assumptions concerning the relative amounts of the species present. They are thus generally applicable to the problem of reannealing a single-stranded probe to a double-stranded DNA.

Fig. 4 shows computer-generated plots of F against R_0 for values of R_0 between 0 and 25. The curves are labeled with various values of the ratio k_1/k_2. It is clear that for reasonable k_1/k_2 the hydridization reaction gives little information for values of R_0 greater than five, since the extent of annealing at saturation is small (less than 20%) and insensitive to R_0. One can verify that hybridization reactions are being done at R_0 values that yield meaningful results by testing several ratios of probe to cellular DNA. If the saturation value is insensitive to changes in R_0 and is less than 30%, the experiments must be done with a smaller ratio of probe to cellular DNA.

TABLE 2. *Calculation of number of copies and relative rate constants*

Hybridization no.	Human DNA (mg)	Globin cDNA (ng)*	$k_2/k_1 =$	F
1	1.8	0.32	$\frac{\log (1-F)}{\log 1R_0 + \log F}$	0.59
2	1.8	0.96	$\frac{\log (1-F)}{\log 3R_0 + \log F}$	0.47
3	1.8	3.2	$\frac{\log (1-F)}{\log 10R_0 + \log F}$	0.30

Reactions paired	R_0	k_2/k_1
1 & 2	0.082	0.295
1 & 3	0.127	0.368
2 & 3	0.113	0.329

In the upper part of the table, the initial amounts of cellular DNA and cDNA are given together with the final extent (F) of hybridization. The expression for k_2/k_1 is also given, expressed in terms of R_0, the initial ratio of cDNA to its complementary sequences in reaction 1. Since reactions 2 and 3 contain, respectively, three and ten times more cDNA, the R_0 is correspondingly higher for those reactions. By equating the expression for any pair of hybridizations, we can solve for R_0 and then for k_2/k_1. When this is done, the results shown in the lower half of the table are obtained. The average value of R_0 is 0.107. Since reaction 1 contains 0.32 ng of cDNA, then it also contains 0.32/0.107 = 2.99 ng of globin sequences complementary to cDNA. Since 0.198 ng represents a single globin gene copy (see legend of Table 1), there are 15(2.99/0.198) globin genes present.

In each of three hybridizations (Tables 1 and 2), we have varied only the concentration of the cDNA; the concentration of the cellular DNA, as well as the temperature, ionic strength, and viscosity, remained unchanged. Since the amount of the cDNA is insignificant compared to the cellular DNA, the reaction conditions were unaltered and, thus, the rate *constants* (but not the rates) for the two reactions are unchanged. By multiplying R_0 by the appropriate constant, we may equate the right-hand side of Eq. [2] for two hybridization curves and solve for R_0 and for the ratio k_2/k_1. We can thus determine the number of copies and the relative rates of the two competing reactions. We analyzed the three hybridizations in this manner and calculated the values of R_0 and k_2/k_1 for each of the three possible pairs. As can be seen in Table 2, the values of R_0 and k_2/k_1 obtained from the three pairs of of curves are in excellent agreement, taking into account a 5% range of error ($\pm 5\%$) in the value of F. From the value of R_0 obtained, we calculate that there are a total of 15 copies of the globin genes. The value for the ratio k_2/k_1 is 0.341, suggesting that the rate constant for reannealing of the cellular DNA globin sequences is 2.9-fold more than that for hybridization between the cDNA to its cellular complement. From the difference in size between the cellular DNA and the cDNA, we would expect k_1/k_2 to be only 1.2. As noted by Straus and Bonner (25), the value of R_0 is quite sensitive to variations in the extent of hybridization, but the value of k_2/k_1 is insensitive to the exact value of R_0.

It has generally been assumed that cDNA and cellular DNA reannealing occurs at the same rate. The results obtained above suggest that this might not be the case. To further test

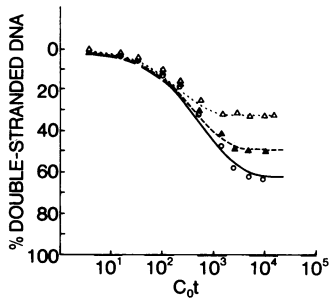

FIG. 5. Computer-generated theoretical hybridization curves satisfying simultaneous equations (25) as described in the *Appendix*. Reactions are as labeled in Table 2. (—) Reaction 1; (---) reaction 2; (····) reaction 3. The points are the actual values taken from Fig. 2.

the validity of the k_2/k_1 and R_0 values, we have determined how well they specify the actual hybridization curves. As noted by Straus and Bonner (25) and by Melli *et al.* (26), the shape of the reannealing curve varies with R_0 and k_2/k_1. If we insert the values obtained above into the system of differential equations that describes the annealing of a single-stranded probe to double-stranded DNA, we can generate the hybridization curves by numerical approximation with a computer. Values of k_1 and k_2 were chosen such that ratio k_2/k_1 = 0.341. The relative shapes of the curves and their positions with respect to one another are fixed by R_0 and k_2/k_1. The positions of the curves relative to the C_0t value are themselves not determined; however, values of k_1 and k_2 may be found by fitting the experimental and theoretical curves. The curves are superimposable upon the experimental data (Fig. 5).

There are several possible explanations for the observed greater rate of reannealing of the cellular globin sequences compared to the hybridization of the cDNA to its cellular complements. One explanation is that some cDNA initially forms weakly bound complexes with γ and δ globin sequences and is thus taken from the pool of free cDNA. Subsequent interaction between these weak complexes and the cellular DNA minus strands might then result in formation of the more stable cellular DNA duplex and the release of the cDNA. The net effect would be to reduce the rate of hybridization of the cDNA relative to renaturation of the cellular sequences.

The fact that the cellular DNA is randomly sheared and contains pieces with both globin and nonglobin sequences, while the cDNA contains only globin information, may also influence the rates. The magnitude of these effects cannot be determined without some knowledge of the types of sequences adjacent to the globin genes in the cellular DNA. If repetitive sequences are adjacent to the native globin DNA, the rate of hybridization of cellular DNA would be markedly increased. In addition, the cDNA may be more highly structured than the randomly broken cellular DNA and, thus would hybridize more slowly. Additional factors that may affect the reaction rates include: the possible thermal degradation of the nucleic acids during the long incubation period; the effect of increased viscosity of the reaction mixture with time since, as the ratio of cDNA to cellular DNA is increased, the hybridization of the cDNA reaches its plateau value at shorter times; and intrinsic rate differences between the various types of globin sequences present.

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