A COMPARISON OF GLOBIN GENES IN DUCK RETICULOCYTES AND LIVER CELLS

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SUMMARY. Synthetic duck globin DNA has been used to compare the number of genes corresponding to globin in duck reticulocytes and liver cells. The results indicate that the number of globin genes does not differ significantly in these organs and suggest, therefore, that neither amplification nor extensive reiteration of these genes occurs as these two cell types become committed to divergent lines of development.

Highly radioactive DNA complementary to globin mRNA (1-3) provides a useful probe with which to determine the relative number of genes corresponding to globin in cells from different organs. Here we report our initial experiments using synthetic globin DNA for this purpose. These experiments view the kinetics of re-association of globin genes in DNA extracted from duck reticulocytes and liver. This type of experiment was devised by Britten and Kohne (4) to determine the relative gene frequency of reiterated and non-reiterated DNA sequences. It has been used, more recently, in studies involving the formation of RNA-DNA hybrids to determine the relative frequency of globin genes in duck erythrocytes (5). While the kinetics of formation of RNA-DNA hybrids cannot be compared directly to the formation of DNA-DNA duplexes, appropriate corrections led Bishop, Pemberton and Baglioni (5) to conclude that there were no more than ten and very likely less than five globin gene copies per duck erythrocyte genome. Previous studies had suggested a much higher reiteration frequency in mouse (6) and chicken (7). Our experiments, involving DNA-DNA duplex formation, indicate that the number of globin genes in the duck reticulocyte does not differ substantially from those in the duck liver and that the globin gene frequency is less than five times that of the non-reiterated portion of the duck genome.

MATERIALS AND METHODS

Purified duck globin mRNA was prepared using procedures described for the purification of rabbit globin mRNA (8). Single stranded, ^{3}H-DNA complementary to purified duck globin mRNA was prepared using the avian myeloblastosis virus reverse transcriptase and an oligo(dT)-primed, purified duck globin mRNA template as previously described for the synthesis of DNA complementary to rabbit globin mRNA (1). This ^{3}H-DNA forms a specific hybrid with polysomal RNA from duck reticulocytes, but does not hybridize with RNA from human or rabbit reticulocyte polysomes or mouse polysomes. Nuclei were isolated from reticulocytes of anemic, phenylhydrazine-treated Muskovie ducks, and from saline-perfused liver of non-anemic ducks (9-11). DNA was prepared by the procedure of Marmur (12), with several modifications (7, 13, 14, 15). Frozen nuclei were thawed and suspended in 2-4 volumes of buffer containing 0.05 M Tris-Cl, pH 8.0 - 0.15 M NaCl - 0.015 M sodium citrate - 2% sucrose - 0.2% sodium dodecylsulfate - 1 mg/ml pronase (DNase-free), and incubated at 37^{o} overnight. $NaClO_4$ was added to a concentration of 1 M and the mixture was extracted with an equal volume of $CHCl_3$:isoamyl alcohol (24:1, v:v) for thirty minutes at room temperature. The aqueous phase was removed after centrifugation, the interphase re-extracted with buffer, the aqueous phases pooled and the DNA precipitated by the addition of two volumes of 95% ethanol. The precipitated DNA was dissolved in buffer containing 0.15 M NaCl-0.015 M sodium citrate (1xSSC) and incubated (37^{o} for 1 hour) with pre-heated (85^{o}, ten minutes) T_1-ribonuclease, 1 unit/ml, and pre-heated pancreatic ribonuclease, 0.1 mg/ml. The reaction mixture was made 0.2% in sodium dodecylsulfate and a second pronase treatment and deproteinization were carried out. The ethanol-precipitated DNA was dissolved in 1xSSC - 0.3 M sodium acetate (pH 7) - 0.0001 M ethylene diaminetetraacetic acid and precipitated with 0.54 volumes of isopropyl alcohol. The precipitate was washed successively with 70%, 80% and 95% ethanol, redissolved in 1xSSC and sheared to a base length of approximately 400 (confirmed by alkaline

sucrose gradient centrifugation) in a Ribi cell fractionator at 50,000 psi (13). The sheared DNA was precipitated with ethanol, and dissolved and dialyzed in buffer containing 0.01 M sodium phosphate pH 6.9 - 0.1 mM ethylenediamine tetraacetic acid.

RESULTS AND DISCUSSION

Reiteration and amplification of ribosomal genes in higher organisms provide biological precedents for suggesting that cells might employ these mechanisms to amplify other genetic information during differentiation (cf. review 14). The avian reticulocyte, committed primarily to the production of globin, provides a convenient system in which to examine this possibility by comparing its genome to avian liver cells which are not involved in globin production (18, 19).

Figure 1 shows the re-association kinetics of ^{3}H-duck globin DNA in the presence of an excess of unlabeled, sheared, denatured double stranded duck reticulocyte or liver DNA. The resulting data can be used to estimate relative gene frequency in two ways: by determination of $C_0t_{1/2}$ (4) and by the determination of the percent of globin ^{3}H-DNA annealed at completion of re-association. The kinetics of re-association are similar in the presence of DNA from reticulocytes and from liver (Figure 1). The half reaction in the presence of reticulocyte DNA occurs at a $C_0t_{1/2}$ of about 730; that in the presence of liver DNA occurs at a $C_0t_{1/2}$ of 540. The difference is not significant in view of the overall precision involved in the determination. These results indicate that there is no significant difference in the number of globin genes in reticulocyte and liver DNA. Similar experiments (not shown) have been carried out in the presence of unlabeled spleen DNA with essentially identical results.

The results also allow us to estimate the reiteration frequency of the duck globin genes. Based on a haploid genome size of 1.15×10^{9} base pairs (23) and the proportionality between genome size and $C_0t_{1/2}$ as determined by

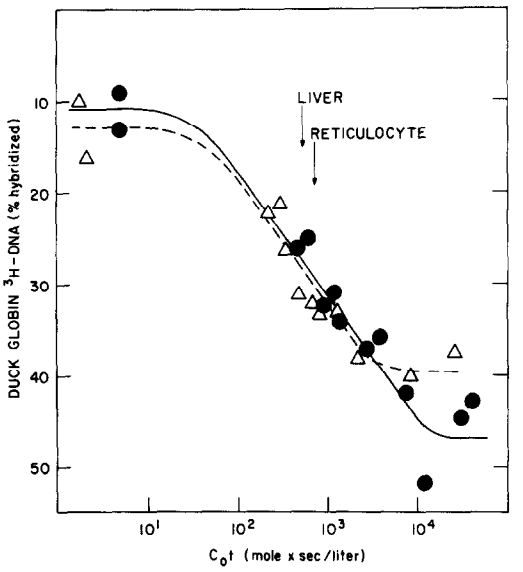

Figure 1. Re-association kinetics of single stranded, ^{3}H-duck globin DNA in the presence of duck reticulocyte or liver DNA. ^{3}H-DNA re-association was carried out at 65° in reaction mixtures containing 0.02 M Tris-Cl, pH 6.9 - 0.7 M NaCl and 1.2 mg/ml sheared, denatured (95°, ten minutes) unlabeled duck reticulocyte DNA or 0.6 mg/ml duck liver DNA. Each reaction mixture also contained 3.6×10^{-3} µgm of single stranded, ^{3}H-duck globin DNA, with specific radioactivity (in dGMP and dCTP) of 5.3×10^7 dpm/µgm. The ^{3}H-DNA is relatively homogeneous, migrating as a molecule containing 250 bases in an alkaline sucrose gradient and is assumed to contain approximately equivalent amounts of DNA complementary to mRNA corresponding to all four duck globins (17). This would provide a 1.2×10^6 fold excess of unlabeled DNA for each radioactive duck globin DNA species. This ratio would be approximately 0.6×10^6 in the case of the reaction mixture containing duck liver DNA. Aliquots were removed at various times and the percent ^{3}H-DNA present as hybrid was determined by its resistance to digestion by nuclease S_1 from Aspergillus oryzae (20-21). C_0t values for each determination were calculated as described by Britten and Kohne (4). C_0t is defined as the product of DNA concentration (Co) and the time (t) of re-association incubation expressed as moles of nucleotides x sec/l. These values, obtained at 0.7 M NaCl, were converted to comparable C_0t values at 0.18 monovalent cation concentration using the relationship determined by Britten and Smith (22). The S_1 nuclease assay was carried out by removing small aliquots (>0.1 ml) from the re-association mixtures and adding them to one ml of buffer containing 0.03 M sodium acetate - 0.1 M NaCl - 0.1 mM zinc sulfate - 10 µgm/ml denatured calf thymus DNA - 10 µgm/ml S_1 nuclease. Control reactions were incubated in the absence of S_1 nuclease. Assay mixtures were incubated at 45° for 45 min and Cl_3CCOOH-precipitable radioactivity was determined. Each assay point represents approximately 600-1000 cpm of ^{3}H-DNA. The experiment shown is a composite of two separate reactions for each unlabeled DNA. Control reactions containing calf thymus or salmon sperm DNA showed no annealing of ^{3}H-DNA at C_0t >10,000.

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optical techniques (4), the $C_0t_{1/2}$ for a unique duck sequence is estimated to be 1500. The observed smaller globin $C_0t_{1/2}$ indicates that there are

2-3 globin gene copies per haploid duck genome. An estimate based on this criterion alone must be qualified in that we have not directly determined the $C_0t_{1/2}$ for non-reiterated duck DNA using the S_1 nuclease assay.

However, we can also estimate the globin gene frequency using an independent calculation especially suited to re-association studies involving the single stranded globin ^{3}H-DNA. This calculation is based on the percent annealing of globin ^{3}H-DNA at very high C_0t when re-association is essentially complete. This parameter depends on the ratios of unlabeled to labeled globin DNA in each reaction. For example, if we add a single stranded globin ^{3}H-DNA sequence to unlabeled, denatured, double stranded duck DNA which contains a precisely equivalent amount of globin DNA, we would expect only 50% of our labeled DNA to anneal. This is so because the labeled sequence would compete with an identical, unlabeled sequence for the complementary unlabeled DNA strand. If the unlabeled DNA contained a ten-fold excess of identical globin sequences, 11 identical sequences (one labeled) would compete for 10 complementary ones and about 90% of the labeled strands would be annealed when re-association is complete. In our experiments, we used a 1.2×10^6 (reticulocyte) and a 0.6×10^6-fold (liver) excess of total unlabeled DNA. At high C_0t, annealing of 36% (reticulocyte) and 26% (liver) of the single stranded ^{3}H-DNA occurred (Figure 1). By this calculation (Table 1), we estimate that the globin gene is represented about 4 times per haploid duck genome as judged in both reticulocyte and liver. (For details of the calculation, see Table 1).

While the above conclusion is roughly in accord with that suggested for duck by Bishop, Pemberton and Baglioni (5) and for mouse embryo by Paul and his associates (personal communication), detailed studies providing further controls are necessary in order to obtain an entirely secure measure of relative globin gene frequency. The results do indicate that cells differentiated for the purpose of producing globin contain essentially the same number of globin genes as cells differentiated for an entirely different

TABLE 1

EXAMPLE OF CALCULATION OF GENE FREQUENCY USING ANNEALING COMPETITION AT COMPLETION OF RE-ASSOCIATION

Given: (a) Unlabeled ds reticulocyte DNA/reaction mixture (r.m.) = 1.2 mg.

(b) ^{3}H-globin, ss DNA/r.m. = 3.8×10^{-6} mg.

(c) No. of bases in ^{3}H-DNA/strand = 250 (see text).

(d) No. of base pairs in duck genome = 1.15×10^{9} (23).

Assume: Synthetic ^{3}H-DNA contains approximately equal amounts of DNA sequences corresponding to the four duck globin subunits (17).

(e) Then, $3.8\times10^{-6}/4 = 0.98\times10^{-6}$ mg of each ^{3}H-DNA sequence present in each r.m.

Let $R^{*} = \dfrac{\text{Unlabeled globin sequence (mg)/r.m.}}{^{3}\text{H-globin sequence (mg)/r.m.}}$

Since (f) only 36% ^{3}H-DNA annealed at completion of re-association (Fig. 1), R = 0.54*

Therefore (g) unlabeled globin sequence/r.m. = 0.54 (f) x 0.96×10^{-6} (e) = 0.52×10^{-6} mg

Let F = Fraction of duck genome present as globin sequences

$$\text{then, } F = \frac{2^{\dagger} \times \text{unlabeled globin sequence (single stranded-mg)/r.m.}}{\text{total unlabeled duck DNA (double stranded-mg)/r.m.}}$$

$$\text{(h) } F = \frac{2\times0.52\times10^{-6}\ \text{(g)}}{1.2\ \text{(a)}} = 0.86\times10^{-6}$$

then, (F) x duck genome size = no. of base pairs as globin sequences in duck genome

(i) or 0.86×10^{-6} (h) x 1.15×10^{9} (d) = 992 base pairs

since no. of base pairs corresponding to ^{3}H-globin sequence = 250 (c), no. of times synthetic globin sequence represented in haploid reticulocyte duck genome =

$$\text{(j) } \frac{992\ \text{(i)}}{250\ \text{(c)}} = 3.99$$

since synthetic sequences represent extensive portions of the globin gene

(k) no. of copies of each duck globin gene/haploid duck genes $\cong$ 4

(l) The value for duck liver, similarly calculated using 26% annealing at completion of re-association with R=0.36* and (a) = 0.6, $\cong$ 4.7.

* These R values are derived using the observed percent annealing and a standard semi-log curve drawn by plotting expected percent annealing at completion of re-association versus R (log scale). As explained in the text, R is a measure of availability of unlabeled complementary strand and competing, identical strand such that if R=1, % annealed = 50; R=5, % annealed = 83; R=0.2, % annealed = 17, etc.

† Since (g) refers to that amount of the unlabeled DNA identical to the ^{3}H-DNA sequence, this must be doubled to account for the complementary strand present in the total unlabeled, double stranded DNA.

purpose. If this result can be taken as typical of organ differentiation, it suggests we focus our attention on the transcriptive and post-transcriptive

reactions in this process. Synthetic globin DNA should prove useful for this purpose as well.

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