REITERATION FREQUENCY OF VITELLOGENIN GENE IN AVIAN LIVER BEFORE AND AFTER ESTRADIOL TREATMENT

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1. Introduction

Several mRNAs have been isolated from eukaryotes and measurement of complementary sequences in total cellular DNA have been carried out by molecular hybridization with either purified mRNA or cDNA [1–20]. With the exception of the histone genes which are present from 10–1000 copies/genome [15–20], the number of complementary sequences of most mRNAs so far tested ranged between 1–5 [1–14]. In the present communication we have used highly purified chicken liver vitellogenin mRNA to synthesize complementary DNA (cDNA) by means of avian myeloblastosis virus (AMV) reverse transcriptase. The size of vitellogenin cDNA was determined by electrophoresis on formamide–polyacrylamide gels and the specificity was judged by the hybridization kinetics with purified vitellogenin mRNA and total polysomal RNA from control and estradiol treated chicks. DNA–cDNA Hybridization kinetics indicate that there is about one copy of vitellogenin gene/haploid genome and that estradiol treatment does not amplify the number of gene copy.

2. Materials and methods

Commercial AMV reverse transcriptase and all four deoxyribonucleotides were obtained from Boehringer Mannheim. d-[^{3}H]CTP (20 Ci/mmol) was purchased from Amersham Radiochemical Centre, England. Oligo $(dT)_{12-18}$ was obtained from Collaborative Research Inc., Waltham, Mass. 02154. White leghorn chicks were used throughout all experiments.

2.1. *Purification of vitellogenin mRNA*

Vitellogenin mRNA was purified from the liver of estradiol treated immature chicks as recently described [21].

2.2. *Purification of AMV and reverse transcriptase*

AMV was propagated in chicken and the infected plasma stored in liquid nitrogen as described elsewhere [22]. Virus was isolated and the reverse transcriptase purified from it following the procedures of Marcus et al. [23]. Enzyme activitiy was eluted from the column with a 0–0.4 M linear KCl gradient. The extent of purification was checked on 7.5% polyacrylamide–sodium dodecyl sulfate (SDS) gels.

2.3. *Preparation of restriction fragments*

λ-[^{32}P]DNA was prepared from *Escherichia coli* C600 (λ C_1 857 S7/λ) which were grown in low phosphate broth containing 2 mCi $H_3{}^{32}PO_4$ [24]. The DNA was digested with endonuclease III from *Haemophilus aegypticus* and the fragments produced were separated on 2.2% acrylamide–0.7% agarose slab-gels. After audioradiography, gel-slices containing fragments of known size were cut out of the gel and the DNA was extracted and purified according to the procedure of Walz [25].

2.4. *Purification of total DNA and total polysomal RNA*

DNA from calf thymus, chicken brain and liver was prepared as described by Sullivan et al. [5]. Total polysomal RNA was obtained as already described [21] except that the polysomes were sedimented through 40% sucrose at 105 000 × *g* for 3 h.

2.5. *Synthesis of vitellogenin cDNA*

Single-stranded complementary DNA was synthesized in a mixture consisting of 0.05 M Tris–HCl, pH 8.3, 0.005 M $MgCl_2$, 0.01 M dithiothreitol, Actinomycin D 20 μg/ml, 10 μg of oligo (dT) and vitellogenin mRNA 100 μg/ml, 0.001 M each of dATP, TTP, dGTP and 0.1 mM [^{3}H]dCTP (20 Ci/mmol). Standard preparations were made in 50–100 μl batches which were started with reverse transcriptase 40 units/ml. After incubation for 1 h at 37°C the reaction was stopped by addition of 0.5% SDS. *E. coli* DNA (50 μg) was added and the RNA template hydrolyzed in 0.2 M NaOH for 5 min at 70°C. The mixture was neutralized and separated on a Sephadex G-50 (fine) column (1 × 70 cm) equilibrated with 0.3 M NaCl–0.01 M sodium acetate pH 5.0 [26]. Only cDNA appearing in the excluded fraction of the column was recovered and precipitated with 2 vol. ethanol at –20°C. The specific activity of the cDNA was about $3.6 \cdot 10^7$ dpm/μg. Aliquots of cDNA were analyzed by electrophoresis on 5.2% polyacrylamide gels containing 98% formamide [27]. Following electrophoresis at 100 V for 7 h at 4°C, gels were sliced and radioactivity measured [27].

2.6. *RNA–cDNA Hybridization*

Samples of RNA ranging from 5 ng – 250 μg were hybridized in silicon treated sterile glass capillaries with 40 pg vitellogenin cDNA (about 1400 dpm) in 10 μl of 0.48 M sodium phosphate, pH 6.8, containing 0.001 M EDTA and 0.1–1% SDS. Capillaries were sealed, heated at 100°C for 5 min to denature all nucleic acids, then incubated at 67°C for the times indicated, ranging from one minute to 10 days. Hybridization was stopped at –80°C. Samples were analyzed for secondary structure by S1 nuclease assay as described by Leong et al. [28]. Each point in a curve represents the average of duplicate determinations. To samples containing very low concentrations of mRNA, 50 μg carrier purified yeast RNA was added/10 μl incubation mixture. The amount of cDNA hybridized for each sample was expressed in percent of the total trichloroacetic acid (TCA) precipitable radioactivity. For the calculation of Crt-values, RNA nucleotides were assumed to have average mol. wt 327.

2.7. *DNA–cDNA Hybridization*

Hybridization of vitellogenin cDNA using vast excess of DNA was carried out exactly as described by Sullivan et al. [5]. The amount of cDNA hybridized was determined after S1 nuclease digestion. For the calculation of Cot values, DNA nucleotides were assumed to have average mol. wt 309.

3. Results and discussion

The size of the cDNA fragments obtained under our incubation conditions was tested on polyacrylamide gels under denaturating conditions. As can be seen in fig.1, vitellogenin cDNA fragments of about 600 nucleotides were obtained. Shortening the incubation time from 1 h to 30 min or increasing the concentration of labeled deoxynucleotides to 1 mM did not have any effect on the size of the cDNA.

3.1. *RNA–cDNA Hybridization*

By comparing the Crt 1/2 obtained for purified vitellogenin mRNA and for total polysomal RNA prepared from the liver of estradiol treated chicks (fig.2A, B) it is possible to measure the fraction of total RNA which is vitellogenin mRNA. We observed about a 500-fold enrichment in vitellogenin mRNA sequences. Assuming that vitellogenin polysomes contain about 50 ribosomes/mRNA [31] and account for 10% of total polysomes, the fraction of vitello-

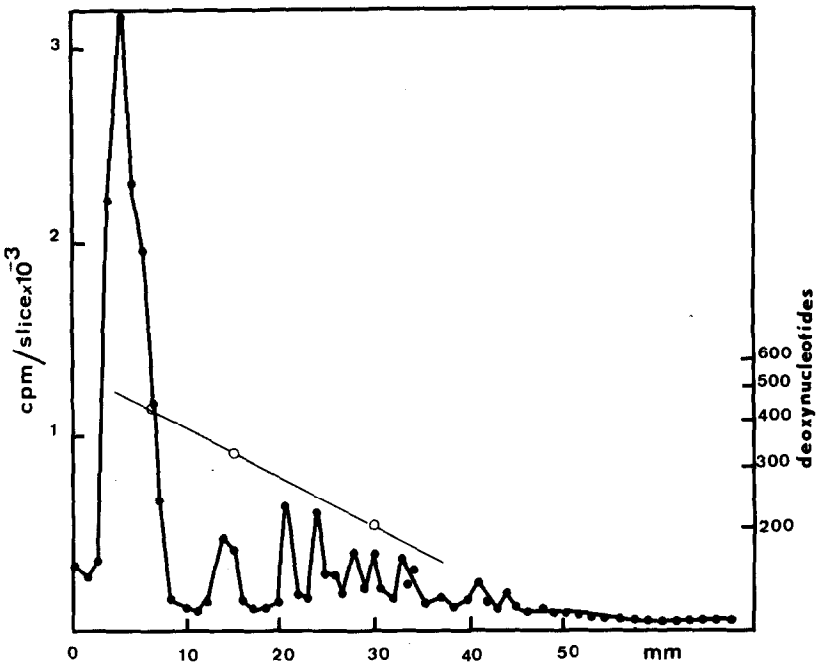

Fig.1. Electrophoresis of vitellogenin [^{3}H]cDNA on 5.2% polyacrylamide gels containing 98% formamide. The calibration curve was established with λ-phage [^{32}P]DNA restriction fragments of 200, 320 and 450 nucleotides.

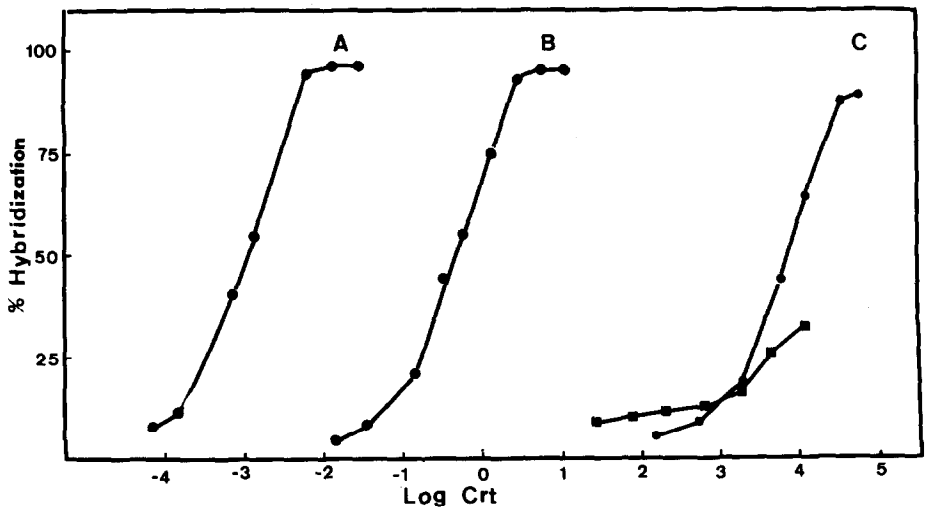

Fig.2. Hybridization kinetics of vitellogenin [^{3}H]cDNA with a vast excess of RNA. A, B, C represent the hybridization of cDNA with purified vitellogenin mRNA, total polysomal RNA from estradiol treated chicks and total polysomal RNA from control chicks respectively. For C the total polysomal RNA was prepared either from new born chicks (60 g) (■———■) or from immature male and female chicks (250 g) (●———●).

genin mRNA in total polysomal RNA and the -fold purification to be expected for pure mRNA can be calculated as follows:

$$\frac{\text{mol. wt vitellogenin mRNA}}{\text{mol. wt rRNA + mRNA}}$$

$$= 10\% \frac{2.4 \cdot 10^6}{50\,(0.7 + 1.6) \cdot 10^6 + 2.4 \cdot 10^6} = 0.2\%.$$

This means that we need about 500-fold enrichment of vitellogenin mRNA in order to get a pure mRNA. As we have seen above, by comparing the Crt 1/2 of polysomal RNA with Crt 1/2 of purified mRNA we achieved in the best case an enrichment of mRNA of about 500-fold. In experiments where the relative purity to vitellogenin mRNA was measured by in vitro translation systems an enrichment of up to 1000-fold was obtained [21]. The discrepancy of this result with the hybridization kinetics presented above can be explained by the possibility that in our cell-free translation system the total polysomal RNA was contaminated with trace amounts of heparin which is known to inhibit the translation of mRNA [32] whereas the purified mRNA was free of contaminating heparin. In addition, we also observed that large quantities of rRNA inhibit the translation of mRNAs.

Hybridization of labeled vitellogenin cDNA with a vast excess of total polysomal RNA (fig.2C) shows that the liver of control chicks contains a low concentration of sequences complementary to cDNA. Whether liver RNA from new born chicks (60 g) or immature male or female chicks (250 g) is used, the same concentration of mRNA is observed, although only about one-third of the total sequences is expressed in new born chicks. These results are in agreement with our previous findings showing that in the liver of immature chicks there are about 20–30 molecules of vitellogenin synthesized/min diploid genome [29]. Under similar conditions Gschwendt and Kittstein [30] found measurable amounts of estradiol receptor in the chromatin of immature chicks. Three days following a secondary stimulating by estradiol (an injection of estradiol given 10 days after the first one) we could detect an increase of about 10 000-fold in the number of complementary sequences hybridizing with vitellogenin cDNA (fig.2B).

3.2. *DNA–cDNA Hybridization*

In experiments where inhibitors of DNA synthesis have been used, it was claimed that the induction of phosvitin synthesis by estradiol (phosvitin is a degradation product of vitellogenin) requires DNA synthesis [33]. It was postulated that the requirement of DNA synthesis could possibly reflect an amplification of vitellogenin gene (or phosvitin) [33]. Using a similar approach we were unable to reproduce these results [34]. The question whether estradiol had any effect on gene amplification could only be answered after hybridization kinetics of vitellogenin cDNA with total cellular DNA were performed. The results in fig. 3 illustrate that whether total DNA of livers from controls or from estradiol-treated chicks (2–3 days following primary stimulation) is used, no difference in the kinetics of hybridization can be shown. Total chicken brain DNA gave similar kinetics whereas total calf thymus DNA did not cross-hybridize with vitellogenin cDNA. One might still argue that the presumptive amplified DNA has been selectively lost during the purification of total DNA. The relative reiteration of vitellogenin gene in the chicken genome can be estimated by comparing the Cot 1/2 of chicken DNA to that obtained for unique sequences in *E. coli*.

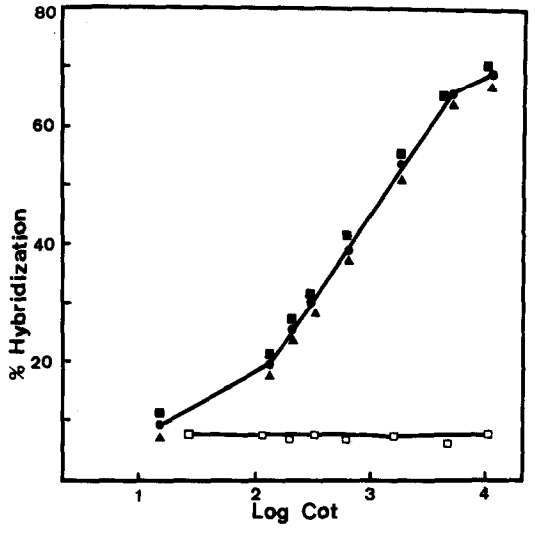

Fig.3. Hybridization kinetics of vitellogenin [^{3}H]cDNA with a vast excess of DNA. DNA was prepared either from chicken brain (▲———▲), liver of control non treated chicks (●———●), liver of estradiol treated chicks (■———■) or from calf thymus (□———□).

Taking a Cot 1/2-value of 3 for unique sequence in *E. coli* DNA [35] and a genome complexity of $4.5 \cdot 10^6$ nucleotide pairs [35], as compared with $9 \cdot 10^8$ nucleotide pairs for the genome of the chicken [4], it is possible to calculate by means of the equation of Melli et al. [36] that unique chicken DNA sequences have a Cot 1/2 of about 600. As shown in fig.3 we found a Cot 1/2 of about 800. This indicates that there is most probably not more than one copy of vitellogenin gene/haploid genome. Using a similar approach, Ryffel et al. [14] found that in Xenopus laevis the renaturation kinetics with cDNA transcribed from estrogen induced RNA (vitellogenin mRNA) and sheared liver DNA gave a Cot 1/2 value typical for single copy gene, thus showing a similarity with the avian system.

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References

[1] Harrison, P. R., Hell, A., Birnie, G. D. and Paul, J. (1972) Nature 239, 219–221.
[2] Bishop, J. O., Pemberton, R. and Baglioni, C. (1972) Nature New Biol. 235, 231–234.
[3] Suzuki, Y., Gage, L. P. and Brown, D. D. (1972) J. Mol. Biol. 70, 637–649.
[4] Harris, S. E., Means, A. R., Mitchell, W. M. and O'Malley, B. W. (1973) Proc. Natl. Acad. Sci. USA 70, 3776–3780.
[5] Sullivan, D., Palacios, R., Stavnezer, J., Taylor, J. M., Faras, A. J., Kiely, M. L., Summers, N. M., Bishop, M. J. and Schimke, R. T. (1973) J. Biol. Chem. 248, 7530–7539.
[6] Bishop, J. O. and Rosbash, M. (1973) Nature New Biol. 241, 204–207.
[7] Harrison, P. R., Birnie, G. D., Hell, A., Humphries, S., Young, B. D. and Paul, J. (1974) J. Mol. Biol. 84, 539–554.
[8] Delovitch, T. L. and Baglioni, C. (1973) Proc. Natl. Acad. Sci. USA 70, 173–178.
[9] Faust, C. H., Diggelmann, H. and Mach, B. (1974) Proc. Natl. Acad. Sci. USA 71, 2491–2495.
[10] Young, B. D., Harrison, P. R., Gilmour, R. S., Birnie, G. D., Hell, A., Humpries, S. and Paul, J. (1974) J. Mol. Biol. 84, 555–568.
[11] Tonegawa, S., Bernardini, A., Weimann, B. J. and Steinberg, C. (1974) FEBS Lett. 40, 92–96.
[12] Rabbitts, T. H. and Milstein, C. (1975) Eur. J. Biochem. 52, 125–133.
[13] Gage, L. P. and Manning, R. F. (1976) J. Mol. Biol. 10, 327–348.
[14] Ryffel, G., Wahli, W. and Weber, R. (1976) Experientia 32, 810.
[15] Jacob, E., Malacinski, G. and Birnstiel, M. L. (1976) Eur. J. Biochem. 69, 45–54.
[16] Kedes, L. H. and Birnstiel, M. L. (1971) Nature New Biol. 230, 165–169.
[17] Wilson, M. C., Melli, M. and Birnstiel, M. L. (1974) Biochem. Biophys. Res. Commun. 61, 404–409.
[18] Jacob, E. (1976) Eur. J. Biochem. 65, 275–284.
[19] Scott, A. C. and Wells, J. R. E. (1976) Nature 259, 635–638.
[20] Weinberg, E. S., Birnstiel, M. L., Purdom, I. F. and Williamson, R. (1972) Nature 240, 225–228.
[21] Jost, J. P. and Pehling, G. (1976) Eur. J. Biochem. 66, 339–346.
[22] Schuerch, A. R. and Wehrli, W. (1977) Manuscript in preparation.
[23] Marcus, S. L., Modak, M. L. and Cavalieri, L. F. (1974) J. Virol. 14, 853–859.
[24] Pirrotta, V., Ptashne, M., Chadwick, P. and Steinberg, R. (1971) in: Proc. Nucleic Acids Research (Cantoni, G. and Davie, D. P. eds) p. 708, Harper and Row, New York.
[25] Walz, A. (1976) Ph. D. thesis, University of Basel, Switzerland.
[26] Ryffel, G. U. and McCarthy, B. J. (1975) Biochemistry 14, 1379–1385.
[27] Jost, J. P. and Pehling, G. (1976) Eur. J. Biochem. 62, 299–306.
[28] Leong, J. A., Garapin, A. C., Jackson, N., Fansher, L., Levinson, W. and Bishop, J. M. (1972) J. Virol. 9, 891–902.
[29] Jost, J. P., Pehling, G. and Baca, O. (1975) Biochem. Biophys. Res. Commun. 62, 957–965.
[30] Gschwendt, M. and Kittstein, W. (1974) Biochim. Biophys. Acta 361, 84–96.
[31] Roskam, W. G., Tichelaar, W., Schirm, J., Gruber, M. and Ab, G. (1976) Biochim. Biophys. Acta 435, 82–90.
[32] Rhoads, R. E., McKnight, G. S. and Schimke, R. T. (1973) J. Biol. Chem. 248, 2031–2039.
[33] Jailkhani, B. L. and Talwar, G. P. (1972) Nature New Biol. 239, 240–241.
[34] Jost, J. P., Keller, R. and Dierks-Ventling, C. (1973) J. Biol. Chem. 248, 5262–5266.
[35] Britten, R. J. and Kohne, D. E. (1968) Science 161, 529–540.
[36] Melli, M., Whitfield, C., Rao, K. V., Richardson, M. and Bishop, J. O. (1971) Nature New Biol. 231, 8–12.