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Chicken lens δ-crystallin gene expression and methylation in several non-lens tissues

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ABSTRACT

RNA sequences coding for the most abundant chicken lens protein, δ-crystallin, were detected at very low levels in day old post hatch chick lung, heart, kidney and liver, and in 6 day embryo headless bodies. The pattern of cytosine methylation within the CCGG sequences of the δ-crystallin genes was also examined and shown to vary in several non-lens tissues, from several stages of development. Embryonic neural retina, which expresses a higher level of δ-crystallin RNA than the above tissues, is no less methylated in the sites studied than the tissues which have no association with the eye, and is actually more heavily methylated than the kidney. Thus no obvious correlation was found between undermethylation and gene expression.

INTRODUCTION

δ-crystallin is the major protein of the bird lens, and the first crystallin to appear in chick lens development. In the 15 day embryo it constitutes 70-80% of the protein of the lens (1), and in the day old post hatch chick lens (21 days of development), when synthesis of the other crystallins is increasing, it constitutes almost 50% of the total protein (2, 3). Two δ-crystallin genes are present in the chick genome (4); they are very similar in sequence and are adjacent on 20kb of the same chromosome (5). Low levels of crystallin RNA have been detected in neural retina and retinal pigmented epithelium from 8 day chick embryos, and somewhat higher levels in the optic cup of 3.5 day embryos (6). These tissues have the capacity to redifferentiate (transdifferentiate) into lentoids, irregular masses of elongated cells with the characteristic features of lens fibre cells, including synthesising large amounts of crystallins (7, 8). Although Jackson et al., (9) and Jones et al., (10), using respectively solution hybridisation and dot hybridisation, failed to detect δ-crystallin RNA in headless embryos, some cross-reactivity with antibodies to crystallins has been detected in kidney and liver, and there has long been controversy about the existence of low levels of crystallin in tissues which have no develop-

-mental or functional association with the eye (11, 12, 8). Using the more senstitive method of hybridisation of a cloned cDNA probe to RNA (Northern) transfers, we have examined nuclear and cytoplasmic RNA from day old post-hatch chick kidney, lung, liver and heart, and 6 day headless embryo, for δ-crystallin RNA sequences. All these tissues are developmentally unrelated to and distant from the eye.

We have also examined the methylation status of CCGG sites in and around the δ-crystallin genes in sperm, blood, liver, lung, kidney and neural retina to establish whether there is a correlation between undermethylation and transcription. Although such a correlation exists for many other vertebrate genes (13, 14, 15, 16), none has been detected in some cases (e.g.17, 18). The reasons for these differences and the role (if any) of DNA methylation in transcriptional control, are not understood (19).

MATERIALS AND METHODS

pM56 and pV89 (Fig. 1), two δ-crystallin cDNA clones (20) were used as probes. Chicks and eggs of inbred line Hy1 (21) were used.

RNA extraction: tissues used were 6 day embryo lens, 6 day embryo headless body, and day old post hatch chick lens, heart, lung, kidney and liver. Cytoplasmic RNA was extracted by the method of Cervera et al., (22). Nuclear RNA was extracted from the pellet by the method of Girard (23). In preliminary experiments, cytoskeletal RNA was also isolated, constituting 1% of total RNA, and was found to be identical in δ-crystallin RNA sequence composition to nuclear RNA (data not shown). RNA associated with high molecular weight complexes was prepared by freeze-thawing tissues to disrupt cells and break nuclei, homogenising in 0.35M sucrose, 10mM Tris pH7.4, 250mM NaCl, 15mM $MgCl_2$, 1mg/ml heparin, 0.1% Triton-X100, filtering through muslin, centrifuging through a sucrose cushion and further purifying as described by Thomson et al., (3). This yields a preparation containing nuclear RNA, polysomal RNA and possibly RNP-associated RNA. Total cellular RNA was prepared by the method of Girard (23).

Electrophoresis of RNA though formaldehyde gels, transfer to nitrocellulose and hybridisation to ^{32}P-labelled pM56 or pBR322 were as described in Bower et al., (20). 10μg/ml of each of polyrA, polyrU, polyrC and polyrG were added to the hybridisation mix to compete out binding of linking regions in pM56 to complementary homopolymer sequences in the RNA preparations. Hybridised filters were washed at 65°C, 4 x 15 min. in 0.1% SDS, 0.1 x SSC (1 x SSC = 0.15M NaCl. 0.015M sodium citrate). The filters were then applied

to Cronex 4 (DuPont) film and exposed at -70°C with a Quanta III (DuPont) intensifying screen.

Oligo-dT cellulose chromatography of RNA: RNA in 0.1% SDS was heated at 70°C for one minute, chilled, adjusted to 0.5M NaCl, 10mM Tris pH7.5, 1mM EDTA, 0.5% SDS, and passed 3 times over oligo-dT cellulose (BRL) which had been equilibrated in the same buffer. The column was washed with 30mls of the same buffer, then polyA+ RNA was eluted in 10mM Tris pH7.5, 1mM EDTA, 0.5% SDS. The eluate was made 0.5M in NaCl and passed again over oligo-dT cellulose as above. The second eluate was adjusted to 0.2M Na acetate and RNA was precipitated with 3 vols. of ethanol. The precipitate was collected by centrifugation and resuspended in H_2O.

Preparation of DNA: adult DNA samples were taken from tissue of a single individual, with the exception of sperm which was pooled from 10 individuals. Other DNA samples were prepared from tissue pooled from several individuals as follows: 1) 16 day embryo neural retina, 10; 2) 8 day embryo neural retina, 40; 3) 16 day embryo liver, 2; 4) 8 day embryo liver, 20; 5) 8 day embryo kidney, 20. All tissues, except sperm, were homogenised in 0.5M EDTA, 0.1M Tris pH 7.5 and filtered through muslin. SDS was added to 0.5%, Pronase to 50 μg/ml and the homogenate rotated at 37°C overnight. It was then extracted 3 times with phenol, once with chloroform, and dialysed for 24 hours in 4 litres of 10mM Tris, 10mM EDTA, 10mM NaCl pH 7.5. RNase A1 (Sigma) was added to 50 μg/ml and after incubation for 3.5 hours at 37°C, SDS was added to 0.5% and Pronase to 50 μg/ml. The solution was again dialysed overnight at 37°C in 4 litres 10mM Tris, 10mM EDTA, 10mM NaCl pH 7.5, then extracted 3 times with phenol/chloroform 1:1, once with chloroform and once with ether. Sodium acetate pH 7.5 was added to 0.3M and 2 vols EtOH were added to precipitate DNA. DNA was washed in 70% ETOH, dried and dissolved in TE (10mM Tris, 1mM EDTA pH 7.5). Any DNA samples which were poorly digested by restriction enzymes were purified on CsCl/ethidium bromide density gradients. After removal of ethidium bromide with amyl alcohol, DNA was precipitated with 3 vols.of 70% EtOH. DNA was washed twice in 70% EtOH, dried and resuspended in TE. Sperm was lysed in 7M Urea, 1% SDS, 0.1M β-mercaptoethanol, extracted 3 times with phenol, once with chloroform and dialysed overnight in 10mM Tris pH 7.5, 10mM NaCl, 10mM EDTA, 0.1% SDS. The DNA was then processed as above, adding SDS and Pronase before the initial phenol extraction, etc.

Hybridisation of probes to DNA (Southern) transfers: 2-6 μg DNA were digested with a 15-30 fold excess of HpaII (Boehringer) or 25-50 fold excess

of MspI (New England Biolabs) under the manufacturer's recommended conditions. λC1857 DNA (0.2 μg) was included as a guide to complete digestion. DNA was run on 1-1.2% agarose gels and transferred to nitrocellulose (Schleicher and Schull) fileter as described by Southern (24), but extending the neutralisation time to 1.5 hours. The transfers were hybridised and autoradiographed as described by Bird et al., (25), with the following alterations. Blots were prehybridised for 16 hours in 20mls 5 x Denhardt's solution (26), in 5 x SSC at 68°C and hybridised for 4 hours at 68°C in 10mls 6 x Denhardt's solution, 6 x SSC, 10% dextran sulphate, 50 μg/ml denatured E.coli DNA; filters were then washed at 60°C in 0.2 x SSC, 0.1% SDS for 20 minutes and twice in 0.2 x SSC.

Plasmid DNA was labelled with ^{32}P by nick translation (27).

RESULTS

The Probes pM56 and pV89 (Fig. 1), two δ crystallin cDNA clones (20) were used as probes. Their relationship to the major δ crystallin mRNA species, which gives rise to both the 48,000 and 50,000 dalton δ crystallin polypeptides (4) is as follows: pV89 corresponds to the 820 nucleotides at the 3' end of the message; pM56 corresponds to the central retion (approx. 750 nucleotides from the 3' end to 1350 nucleotides from the 3' end). The cDNA insert in pM56 contains only coding sequence.

Detection of RNA Transcripts. Northern transfers of RNA fractions from a variety of tissues were hybridised to pM56 (Fig. 2). Heart, lung, liver, kidney and lens cytoplasmic RNA from day old post hatch chick all showed a diffuse band of 6kb. The major lens band at 2kb was not detected in these tissues (Fig. 2a, lanes 1,3,5,7). Day old chick nuclear RNA from heart, lung and liver showed a faint band at 6kb and a very diffuse band containing material from 9kb to about 22kb (Fig. 2a, lanes 2,4 and 6); nuclear RNA from day old chick lens gave bands at 9, 6 and 3.8kb, and diffuse labelling in the region of 15-22kb (Fig. 2a, lane 8); distinct bands at 2 and 3.1kb were visible after shorter exposures than shown here. Both cytoplasmic and nuclear RNA from headless 6 day embryo chick showed hybridisation of pM56 to bands of 2 and 6kb (Fig. 2b, lanes 1 and 2). The relative proportions of the two bands in headless embryo and lens were different. RNA from high molecular weight complexes from day old chick lung, heart, kidney and liver after long exposure all showed a band at 2kb; lung and kidney had an additional band at 9 kb and lung at 22kb (Fig. 2c, lanes 3-6).

6 day embryo lens RNA showed relatively more hybridisation to the 2kb

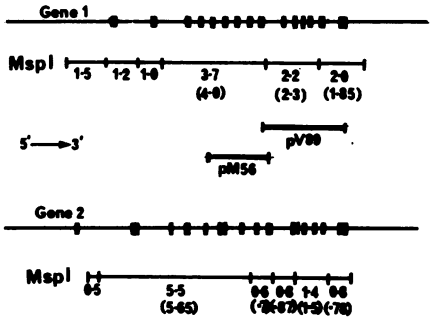

Fig. 1. δ-Crystallin genes 1 and 2 with the MspI fragments as mapped by Jones et al., (10) shown below each gene.
The sizes of the MspI fragments detected by Jones et al., (10) are in kb. The numbers in the brackets are the fragments detected and sized by us, and allotted to the map positions. The locations of exons are indicated by the black boxes on genes 1 and 2, and the approximate regions of exon homology to the two cDNA clones pV89 and pM56 are also shown. The cumulative size of the exon coding region for gene 1 is approx. 2kb (4, 5) which is the size of the major fully processed δ-crystallin mRNA.

band in the nuclear RNA than in the cytoplasmic RNA (Fig. 2d, lanes 1 and 2), and the nuclear RNA showed major bands of hybridisation at 22kb (comigrates with the largest λ EcoRI fragment) and >30kb (not yet accurately sized). Fainter bands were detected in the nuclear RNA at 6 and 9kb. After long exposure, other bands corresponding to those found in day old lens nuclear RNA were seen (not shown). ^{32}P-labelled pBR322, the plasmid moiety of pM56, gave no detectable hybridisation to day old chick lens RNA after prolonged exposure (Fig. 2e).

Northern transfers of polyA+ RNA from day old chick lung, kidney, heart, liver (all 15 μg/lane) and lens (150ng/lane) hybridised to pM56 (Fig. 3, lanes, 1-5) all showed a diffuse band at about 6kb. In addition, lung and kidney had a band at 3.8kb, heart and liver had a band at 4.2kb. Bands were also seen with heart (at 9kb) and kidney (2.5, 2.0 and 0.8kb). The overall level of hybridisation to kidney polyA+ RNA was somewhat higher than to lung, heart and liver, but less than to lens RNA which was present here at 1% of the concentration of the other tissues' RNAs. A lane containing lens polyA- RNA (the flow-through from oligo-dT cellulose) was run on the same gel, cotransferred and hybridised to a ^{32}P-labelled cloned Xenopus ribosomal DNA repeat, a gift of C. Phillips (Fig. 3, lane 6). The bands corresponding to ribosomal RNA did not comigrate with the bands in various tissues hybridising to pM56.

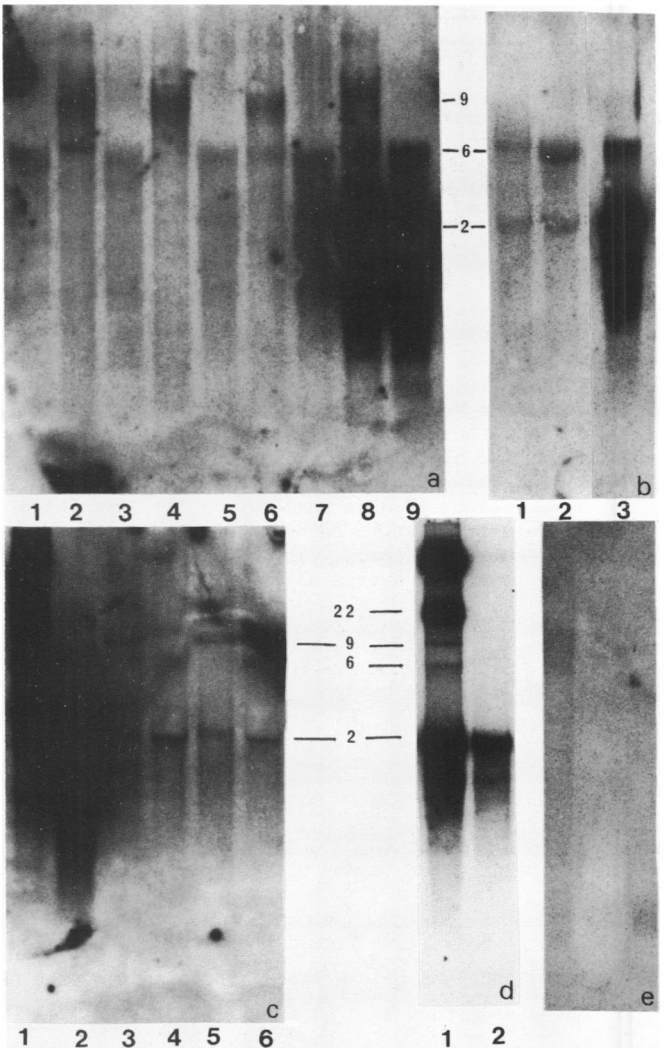

Fig. 2a Autoradiographs of RNA transfers hybridised to ^{32}P-pM56. 15μg RNA per lane. 10 day exposure. All RNA from day old hatched chick.
Lane 1 - heart cytoplasmic RNA; Lane 2 - lung nuclear RNA;
Lane 3 - lung cytoplasmic RNA; Lane 4 - liver nuclear RNA;
Lane 5 - liver cytoplasmic RNA; Lane 6 - kidney nuclear RNA;
Lane 7 - kidney cytoplasmic RNA; Lane 8 - day old hatched chick lens nuclear RNA;
Lane 9 - day old hatched chick lens cytoplasmic RNA. Size given in kilobases.

Fig. 2b Autoradiographs of 15μg/lane of transferred and hybridised RNA as in Fig. 2a. Lane 1:6 day embryo headless body nuclear RNA;

Lane 2: 6 day embryo headless body cytoplasmic RNA; Lane 3: day old hatched chick lens cytoplasmic RNA.

Fig. 2c Autoradiographs of 15μg/lane of transferred and hybridised RNA as in Fig. 2a except exposure was for 28 days.
Lane 1 - λ size marker; Lane 2 - day old hatched chick lens cytoplasmic RNA;
Lane 3 - total high molecular weight complexed lung RNA.
Lane 4 - total high molecular weight complexed heart RNA.
Lane 5 - total high molecular weight complexed kidney RNA.
Lane 6 - total high molecular weight complexed liver RNA.

Fig. 2d. Autoradiographs of 15μg/lane of transferred and hybridised RNA as in Fig. 2a.
Lane 1 - 6 day embryo lens nuclear RNA.
Lane 2 - 6 day embryo lens cytoplasmic RNA.

Fig. 2e Autoradiograph of 15 μg/lane of transferred day old hatched chick lens cytoplasmic RNA hybridised to ^{32}P-pBR322. 28 day exposure.

Methylation of the δ-Crystallin Genes The methylation pattern exhibited by individual genes has been examined using the isoschizomers HpaII and MspI, which are sensitive and insensitive respectively to methylation at the internal cytosine in their recognition sequence, CCGG (28, 29, 30). The methylation status of this sequence within the δ-crystallin gene regions (Fig. 1) was assessed in several tissues. In contrast with previous authors (31, 10), we found it necessary to use a considerable excess of HpaII and MspI. Our criteria for completeness of digestion were 1) limit digestion of the internal DNA; 2) no change in the restriction pattern after redigestion with fresh enzyme.

Hybridisation of pV89 (homologous to the 3' half of the δ crystallin genes) to MspI digested sperm and blood DNA (Fig. 4, lane B and D) detected nine DNA bands: 2.3, 1.85, 1.5, 0.95, 0.87, 0.78, 0.7, 0.5, and 0.4 kb. Identical digest patterns were found with all somatic tissues examined from all stages of development. The 2.3 and 1.85 kb bands are presumed to correspond to the mapped 2.3 and 2.0 kb bands at the 3' end of gene I (10), shown in Fig. 1. Likewise our 1.5, 0.87, 0.78, and 0.7 kb bands probably correspond to the 1.4, 0.8, 0.8 and 0.6 kb fragments at the 3' end of gene II (10), shown in Fig. 1. The 0.5 and 0.4 kb were not mapped or detected in hybridisations by Jones et al., (10), and could easily have been missed due to their relatively very faint hybridisation signals. The 0.95 kb was also detected by Jones et al., (10), but remains unmapped. We have previously reported (32) two MspI bands at 4.0 and 5.65 kb, using as probe pM56, which is homologous to the central region of the two δ crystallin genes (Fig. 1). These bands are presumed to correspond to the 3.7 and 5.5 kb detected by Jones et al., (10), which are located in the middle of gene I and the middle and 5' regions of

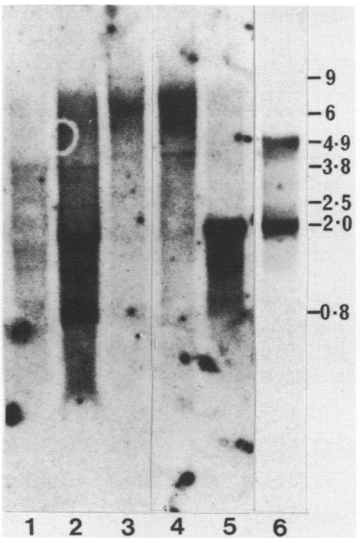

Fig. 3. Autoradiographs of transferred and hybridised RNA as in Fig. 2a, except exposure of lanes 1-5 was 14 days, lane 6 was 3 days. In lanes 1-4, 15μg/lane of polyA+ RNA from several day old hatched chick tissues was loaded. In lane 5, 150ng of polyA+ RNA from day old hatched chick lens was loaded. In lane 6, 3μg of polyA- RNA (unbound fraction from oligo-dT cellulose) was loaded. All lanes were run on same gel and transferred together. Lanes 1-5 were hybridised to ^{32}P-labelled pM56. Lane 6 was hybridised to ^{32}P-Xenopus laevis cloned ribosomal DNA.
Lane 1 - lung poly A+ RNA; Lane 2 - kidney polyA+ RNA;
Lane 3 - heart poly A+ RNA; Lane 4 - liver polyA+ RNA;
Lane 5 - lens polyA+ RNA; Lane 6 - lens poly A- RNA

gene II respectively (Fig. 1).

With the exception of sperm DNA all HpaII digests show fragments of 3.0, 3.25, and 6.3 kb. A 2.3 kb fragment present in adult blood (lane C), lung, (lane J), liver (lane E) and kidney (lane H), HpaII digests is found at much lower levels in embryonic kidney and neural retina and is undetectable in embryonic liver. A 0.87 kb HpaII fragment present in adult blood (lane C) and kidney DNA (lane H) is not detected in adult liver (lane E) or lung, (lane J) or in any embryonic tissue examined. Both adult and embryonic kidney (lane H, I) differ from all the other tissues tested in showing HpaII bands at 1.85 and 1.6kb. There are therefore at least two sites in kidney DNA which are hypomethylated in comparison to the other somatic tissues examined. Small quantities of HpaII bands at about 4.6 and 4.3 kb were detected in all tissues, but showed up more strongly in digests of kidney

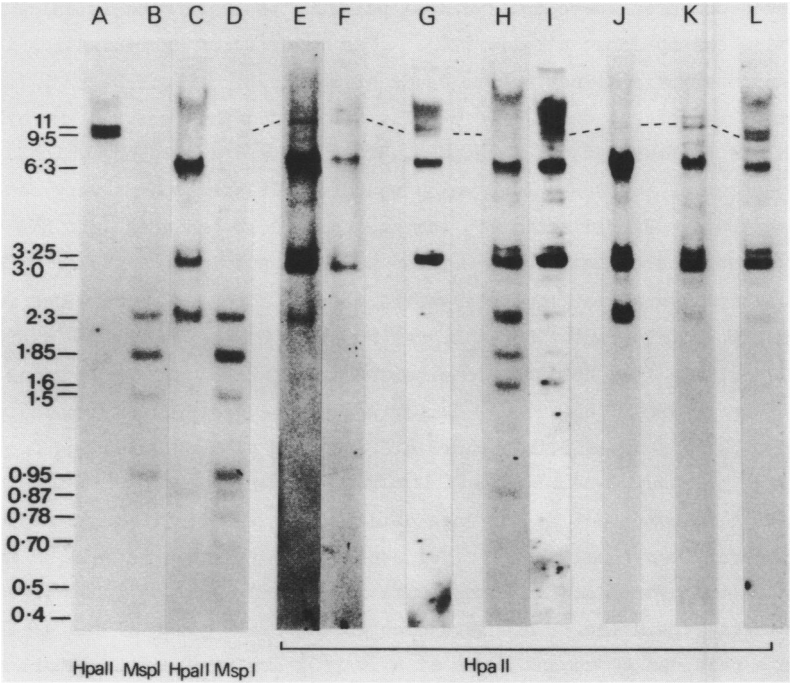

Fig. 4 ^{32}P-labelled pV89 was hybridised to a nitrocellulose transfer of MspI or HpaII digests of DNA extracted from various chicken tissues.
Lane A and B - sperm DNA; Lane C and D - adult blood DNA;
Lane E - adult liver DNA; Lane F - 16 day embryo liver DNA;
Lane G - 8 day embryo liver DNA, Lane H - adult kidney DNA;
Lane I - 8 day embryo kidney DNA; Lane J - adult lung DNA;
Lane K - 16 day embryo neural retina;
Lane L - 8 day embryo neural retina.
A lane carrying size marker λ EcoR1 DNA was separately hybridised to ^{32}P-λ DNA (not shown). Sizes in kilobase pairs. The figure is a composite of three DNA transfers. The largest fragments do not align perfectly and the dotted line indicates their relative position.

DNA.

HpaII digests of sperm DNA (lane A) reveal the presence of only two high molecular weight bands, which are of similar length to the two δ-crystallin genes (10). HpaII digests of all the embryonic tissues examined reveal small quantities of these two bands, but they are undetectable in the adult tissues examined with the exception of liver DNA. No difference in MspI digest patterns was observed between tissues. Parallel hybridisations to pBR322 alone failed to produce any signal.

We have compared these HpaII digest patterns to that found in chick embryonic lens by Jones et al., (20). The majority of hybridisation in adult and embryonic kidney was to bands of 6.3, 4.3, 3.25, 3.0, 2.3, 1.85, and1.6kb. With lens DNA, Jones et al., (10) found that most of the hybridisation was to bands at 6.5, 4.4, 4.0, 3.1, 3.0, 2.0 and 1.5kb, which, with the exception of the 2.3kb band which we detected, corresponds very closely to kidney DNA. Hybridisation to the 4.4 and 4.0 kb bands in lens DNA was relatively stronger than to the 4.6 and 4.3 kb bands in kidney DNA. The 2.3 kb band which hybridised moderately strongly in kidney DNA hybridised weakly in lens DNA. This variation in signal strength may be due to the different probes used, the 1300 bp cDNA clone (pδCr2) of Jones et al., (10) being larger than pV89 (820 bp; 20). Comparison of the restriction maps of pδCr2 (4) and pV89 (20) reveals that pδCr2 differs from pV89 in extending further into the 5' regions of the δ crystallin mRNA coding sequences. This observation is borne out by the data obtained by hybridisations to mapped MspI fragments mentioned above. Yet overall the strength as well as the pattern of the hybridisation signal with kidney and lens HpaII DNA digests were remarkably similar. Discrepancies in the exact sizes of the HpaII fragments obtained by us and Jones et al., (10) are very small and are easily within the margin of error for molecular weight estimations.

DISCUSSION

Transcription of δ-Crystallin Genes The δ-crystallin cDNA probe pM56, hybridises with high specificity to only two sequences in Southern transfers of EcoR1 digests of chick genomic DNA; these sequences correspond to the two δ-crystallin genes (20). No other EcoR1 fragments are labelled even when the hybridisation and washing temperature is lowered from 65°C to 60°C, and washes are at low stringency, i.e. 2 x SSC (unpub. data). The two bands detected by pM56 are 10.5 and 3.25kb (20), quite different from the major bands detected by either end-labelled chick robsomal RNA or a cloned Xenopus laevis rDNA repeat, which are 18, 13.5, 14.4 and 0.5kb (data not shown). Since there are about 200 copies of the ribosomal genes in chick (33), one would expect that weak cross-reaction between pM56 and a sequence in ribosomal DNA would be detected under these conditions, unless hybridisation was to an unusual sequence present in only a small number of the total rDNA gene copies. It seems unlikely that a rare ribosomal gene would make a major contribution to the rRNA population, and give rise to artifactual bands by a weak cross hybridisation to pM56. In the experiments presented here,

hybridisation and washing conditions were highly stringent, and hybridisation mixes contained 10μg/ml of each of the four polynucleotides. ^{32}P-labelled pBR322 gave no hybridisation to day old lens cytoplasmic RNA. Thus it seems probably that the hybridisation detected between pM56 and RNA preparations from non-lens tissues indicates the presence of δ-crystallin RNA sequences, in these tissues, though at a very low level relative to lens. In addition, 1) of the bands detected by pM56, only the 2kb band comigrates with a major ribosomal band; yet it remains undetected in Fig. 2a hybridisations (lanes 1, 3,5,7), which all contain rRNA. 2) The bands complementary to pM56 in the polyA+ RNA (Fig. 3) are not all detected in total RNA and are therefore preferentially present in the polyA+ fraction. 3) All the non-lens tissues were contaminated by a small amount of blood, but the possibility that this contaminant is the source of the δ-crystallin RNA sequence is unlikely, because the pattern of the bands of poly A+ RNA is different between tissues; also blood would have to contain a high level of δ transcripts to be detected at this level, since it is only present in trace amounts in the tissues.

The proportion of total cytoplasmic and total nuclear RNA (Fig. 2a) hybridising to pM56 is similar in day old kidney, liver, heart and lung; the pattern of transcripts is also similar. However, they are quite different from the pattern of transcripts found in day old lens nuclei (Fig. 2a) and 6 day embryo lens nuclei (Fig. 2d). There are quantitative differences between 6 day embryo lens nuclei, where δ gene transcription is at a maximum (1) and day old chick lens nuclei where δ transcription is falling (1). Lens transcripts at the two stages appear to be qualitatively more similar to each other than to the other tissues. The highest molecular weight precursors found at 6 days are not present in clearly detectable amounts in the day old post hatch lens, but this may be due to faster processing of the primary transcript, which is being produced in smaller amounts at this stage. There is a lag between increased transcription and increased processing of δ-crystallin RNA in 6 day embryo lenses (34).

When RNA associated with high molecular weight structures was purified,it gave sharper bands of hybridisation to the probe, and the main band detected was 2kb, although the exposure was much longer. This suggests that most of the δ-crystallin RNA sequence in day old non-lens tissue may not be associated with either processing complexes or polysomes, but dissociated from any functional complexes and subject to gradual degradation. Some δ crystallin RNA however, does seem to be undergoing a more orderly processing to the normal δ-crystallin mRNA size of 2kb. The headless embryo RNA seems to

belong to this class. In all cases the amount of δ-crystallin RNA sequence is very small compared with lens.

After passage over oligo-dT cellulose, which enriches 20-100 fold for poly A+ sequences, we detect in non-lens tissues, some of the diffuse 6kb band detected in total cytoplasmic RNA; there are several new bands in liver; kidney and lung, including a major 2kb band in kidney. The enrichment for δ-crystallin RNA sequences is not very great, implying that the new sequences which are detected in the poly A+ RNA are being turned over rapidly. Since high molecular weight-complexed RNA from all tissues examined contains a 2kb band, but only kidney has a 2kb band of poly A+ RNA, it is possible that not all transcripts are being polyadenylated.

All of the δ crystallin RNA sequence cloned in pM56 codes for protein (see Results), therefore all of the tissues examined contain at least part of the δ-crystallin coding sequence. We do not know whether one or both δ-crystallin genes are being transcribed. It has not yet been established whether any of this RNA is translated into protein; however, traces of crystallin antigenicity were previously detected in these tissues (12). It is not clear what function, if any, such a protein would have outside the lens. Conceivably, different splicing of transcripts from the δ-crystallin gene region might give rise to mRNAs for completely different polypeptides, as has been shown in several other cases including the β-neo-endorphin gene (35), the calcitonin gene (36) and the Aplysia egg-laying proteins (37). The possibility remains, however, that these transcripts are due to a permissible degree of 'leakiness' in the control of transcription and incomplete processing, and that they have no functional significance.

Methylation Pattern of the δ-Crystallin Genes Differences in HpaII fragment patterns between tissues reflect the tissue specificity of the methylation pattern of defined CCGG-sites within the δ-crystallin genes. These genes are heavily methylated in sperm DNA but are less heavily methylated in somatic tissues where the patterns vary. In addition, there appear to be differences in the HpaII fragment patterns between embryo and adult DNAs of liver and kidney. This result might have been due to polymorphism, but if even one of the 20 individuals used to make 8 day embryonic liver and kidney DNAs had produced the extra fragments we detect in adults, we would expect to have detected them. We conclude that at least two HpaII sites in liver and perhaps six in kidney have become unmethylated during development. The significance of this observation is unclear. Polymorphism might also have been invoked to account for the two high molecular weight bands (11, 9.5 kb)

present in HpaII digests of embryonic somatic tissue DNAs. However, since these bands are also faintly present in adult liver DNA from a single individual, it is more likely that they are due to differences between cells within a tissue.

In contrast, with the variations in methylation pattern of the δ-crystallin genes between somatic tissues (Fig. 3), the RNA transfers indicate that gene transcription occurs at a similar low level in tissues developmentally unrelated to the eye. In addition, the δ-crystallin genes in embryonic neural retina, which are known to be expressed at a relatively high level (9) (though still low relative to lens), are more methylated than those in other somatic tissues. The least methylated tissue DNA, kidney (Fig. 3) which expresses δ crystallin at a very low level (Fig. 2), shows a very similar methylation pattern to 5, 10 and 15 day embryo lenses (10) which are transcribing the δ crystallin genes at a very high level.

Many vertebrate genes including those for human (13), rabbit (38) and chicken (39) β-globin, chicken α-globin (16), chicken egg proteins (14), and mouse metallothionein-1 (15), not only exhibit tissue specific patterns of DNA methylation, but the extent of this methylation also correlates inversely with transcription. We do not observe a correlation between undermethylation and transcription for the δ-crystallin genes except perhaps in sperm whose δ-crystallin genes are heavily methylated and presumably not transcribed. The high level of methylation found in sperm δ-crystallin genes has also been found in other chicken genes such as β-globin (38, 40), α-globin (16) and the egg protein genes (14) in sperm.

We cannot preclude such a correlation between undermethylation of specific sites and transcription for various reasons. In common with previous workers, (10) we lack a 5' probe and cannot therefore examine this region of the genes. However, it does appear that for many genes, variably methylated CpG sites are not confined to the 5' end of the gene. All CCGG sites in mouse metallothionein-1 genes (15) are methylated or unmethylated concurrently depending on the transcriptional status of the gene. A large unmethylated domain exists around the transcribed chicken α-globin genes which extends 1kb beyond the 3' end of the coding regions (41). Variably methylated HpaII sites that correlate with transcription are also found throughout the human, rabbit and chicken β-globin genes (13, 38, 39) and one such site has been found at the 5' end of the chicken α2(1) collagen gene (42). Only rat albumin (43) and chicken vitellogenin (17) genes posess variably methylated sites which are confined to the 5' end. While undermethylation may be a

necessary but not sufficient condition for transcription to occur, it remains possible that only a few critical CpG sites may be involved.

Three other caveats should now be mentioned with regard to these conclusions. Firstly, the restriction enzymes, HpaII and MspI, can only be used to analyse a small proportion of methylatable CpGs, namely CCGG sequences. Secondly it is not possible to detect very closely spaced HpaII sites since small DNA fragments bind inefficiently to nitrocellulose. Thus variably methylated CpG sites that correlate with transcription might be overlooked. Finally, in any one tissue, the proportion of cells actively transcribing δ-crystallin is not known. If for example, this proportion was small in neural retina, no correlation between undermethylation and transcription would be detected.

In conclusion, the methylation patterns of the chick δ-crystallin genes bear more resemblance to human growth hormone and chorionic somatomammotropin genes (18) and Xenopus vitellogenin genes (17), where tissue-specific differences are observed but no correlation with transcription can be detected. Whether, as these authors (17, 18) have suggested, the methylation pattern correlates more closely with the state of differentiation of the tissue rather than with its transcriptional status, remains to be seen.

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REFERENCES

1. Piatigorsky, J., Beebe, D., Zelenka, P., Milstone, L.M. and Shinohara, T. (1976) INSERM, 60, 85-112.
2. Genis-Galvez, J.M., Maisel, H. and Castro, J. (1968) Exp. Eye Res., 7, 593-602.
3. Thomson, I., Williamson, R., Burns, A., Truman, D.E.S. and Clayton, R.M. (1978) Exp. Eye Res., 26, 351.
4. Bhat, S.P. and Piatigorsky, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3299-3303.
5. Yasuda, K., Kondoh, H., Okazaki, T.S., Okada, T.S. and Shimura, Y., (1982) Proc. Vth Int. Cong. Eye Res., Eindhoven, p32.
6. Clayton, R.M., Thomson, I. and de Pomerai, D.I. (1979) Nature 282, 628-629.
7. Okada, T.S. (1980) Curr. Top. Dev. Biol., 16, 349.
8. Clayton, R.M. (1982) In British Society for Cell Biology, Symposium 4, eds., Yeoman and Truman, Cambridge University Press, 83-120.
9. Jackson, J.F., Clayton, R.M., Williamson, R., Thomson, I., Truman, D.E.S. and de Pomerai, D.I. (1978) Dev. Biol., 65, 383-395.

10. Jones, R.E., De Feo, D. and Piatigorsky, J. (1981) J. Biol. Chem. 256, 8172-8176.
11. Barabanov, V.M. (1982) Doklady Akad. Nauk, S.S.S.R., 262, (6) 1491-1494.
12. Clayton, R.M., Campbell, J.C. and Truman, D.E.S. (1968) Exp. Eye Res., 13, 11-29.
13. Van der Ploog, L.H.T. and Flavell, R.A. (1980) Cell, 19, 947-958.
14. Mandel, J.L. and Chambon, P. (1979) Nucleic Acids Res., 7, 2081-2103.
15. Compere, S.J. and Palmiter, R.D. (1981) Cell 25, 233-240.
16. Haigh, L.S., Owens, B.B., Hellewell, S. and Ingram, V.M. (1982) Proc. Nat. Acad. Sci. U.S.A. 79, 5332-5336.
17. Wilks, A.F., Cozens, P.J., Mallaj, I.W. and Jost, J. (1982) Proc. Nat. Acad. Sci. U.S.A. 79, 4252-4255.
18. Hjelle, B.L., Phillips, J.A. and Seeburg, P.H. (1982) Nucleic Acids Res. 10, 3459-3474.
19. Razin, A. and Riggs, A.D. (1980) Science 210, 604-610.
20. Bower, D.J., Errington, L.H., Wainwright, N.R., Sime, C., Morris, S. and Clayton, R.M. (1981) Biochem. J., 204, 339-344.
21. Clayton, R.M. (1975) Genet. Res. (Camb.) 25, 79-82.
22. Cervera, M., Dreyfuss, G., and Penman, S. (1981) Cell, 23, 114-120.
23. Girard, M. (1967) in "Methods in Enzymology" XIIA, eds., Grossman and Moldave, Academic Press, N.Y. pp581-588.
24. Southern, E.M. (1975) J. Mol. Biol. 98, 503-517.
25. Bird, A.P., Taggart, M.H. and Smith, B.A. (1979) Cell, 17, 889-901.
26. Denhardt, D. (1966) Biochem. Biophys. Res. Commun. 23, 641-646.
27. Maniatis, T., Jeffrey, A. and Kleid, D.G. (1975) Proc. Nat. Acad. Sci. U.S.A. 72, 1184-1188.
28. Mann, M.B. and Smith, H.O. (1977) Nucleic Acids Res., 4, 4211-4221.
29. Sneider, T.W. (1980) Nucleic Acids Res., 8, 3829-3840.
30. Bird, A.P. (1978) J. Mol. Biol., 118, 27-47.
31. Schmookler Reis, R.J., Goldstein, S. (1982) Nucleic Acids Res., 10, 4293-4303.
32. Errington, L.H., Cooper, D.N. and Clayton, R.M. (1983) Differentiation (in press).
33. Shoyab, M., Markham, P.D. and Baluda, M.A. (1974) J. Virol., 14, 225-230
34. Bower, D.J., Errington, L.H., Pollock, B.J., Morris, S. and Clayton, R.M. (1983) EMBO J., 2, (in press).
35. Kakidani, H., Furutani, Y., Takahashi, H., Noda, M., Morimoto, Y., Hirose, T., Asai, M., Inayama, S., Nakanishi, S. and Numa, S. (1982) Nature 298, 245-249.
36. Amara, S.G., Jonas, V. and Rosenfeld, M. (1982) Nature, 298, 240-244.
37. Scheller, R.H., Jackson, J.F., McAllister, L.B., Rothman, B.S., Mayeri, E. and Axel, R. (1983), Cell 32, 7-22.
38. Shen, E.K.J. and Maniatis, T. (1980) Proc. Nat. Acad. Sci. U.S.A. 77, 6634-6638.
39. McGhee, J.D. and Ginder, G.D. (1979) Nature (London) 280, 419-420.
40. Waalwijk, C. and Flavell, R.A. (1978) Nucleic Acids Res., 5, 4631-4641.
41. Weintraub, H. and Groudine, M. (1981) Cell 24, 333-344.
42. McKeon, C., Ohkubo, H., Pastan, I., and de Crombrugghe, B. (1982) Cell 29, 203-210.
43. Ott, M-O., Sperling, L., Cassio, D., Levilliers, J., Sala-Trepat, J. and Weiss, M.C. (1982) Cell 30, 825-833.