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Adult chicken α-globin gene expression in transfected QT6 quail cells: evidence for a negative regulatory element in the αD gene region

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ABSTRACT

The chicken adult α-globin genes, αA and αD, are closely linked in chromosomal DNA and are coordinately expressed *in vivo* in an approximate 3:1 ratio, respectively. When subcloned DNAs containing one or the other gene are stably transfected into QT6 quail fibroblasts, the αA-globin gene is expressed at measurable RNA levels, but the αD gene is not. The αA gene expression can be considerably increased by the presence of a linked Rous sarcoma virus long terminal repeat enhancer, but that of the αD gene remains undetectable. Transfection with subclones containing both genes, either in *cis* or in *trans*, leads to considerably reduced αA RNA levels and still no observable αD gene expression. Transfection with deleted subclones suggests that maximal expression levels in this system require the αA-globin gene promoter, as opposed to that of the αD gene, but that such expression is greatly reduced by one or more DNA sequences which lie approximately 2,000 base pairs upstream of the αA gene, within the body of the αD gene.

INTRODUCTION

Globin gene expression has served as a useful model system in which to study transcriptional regulation in higher eukaryotes. Regulatory elements present in and around mammalian α- and β-globin genes (1–11) and, to a lesser extent, the chicken globin genes (12–16) have been identified primarily by transfecting cloned globin gene DNA (*in vitro*-mutated or wild type) into cultured cells, or by use of transgenic animal systems (17–22), for the examination of the level of expression of the exogenous gene(s). Transient and stable transfection experiments of this type have provided evidence for two types of cis-acting DNA sequences: constitutive elements consisting of sequences capable of functioning in both nonerythroid and erythroid cells (3,7–9, 23) and erythroid-specific sequences that function solely in erythroid lineage cells (1–13). Such elements include both enhancers and silencers which affect transcription in positive and negative manners, respectively, in both a position- and orientation-independent fashion. (24–32). Cis-acting elements of mammalian globin genes that function constitutively have been studied in HeLa (3, 7–9, 23), COS (9), 293 (9), 3T6 (6), and murine erythroleukemia (MEL) (3, 5) cells, among others. Erythroid-specific DNA regulatory sequences have been identified in mammalian globin genes primarily in MEL cells (1–5, 10, 33, 34) and, to a lesser extent, in transgenic mice (17–20, 35–37). Constitutive regulatory elements have not been as carefully delineated in avian globin genes. However, erythroid-specific enhancer elements have been identified downstream of both the chicken β-globin gene (11, 12, 15, 38, 39) and the chicken αA-globin gene (40).

The studies reported here were undertaken to investigate constitutive regulatory sequences of the chicken α-globin genes. Avians are unusual in that they have two closely-linked adult α-globin genes, αA and αD, which are very different in sequence, and which thus presumably result from an ancient globin gene duplication (41,42). Both genes are expressed at low levels in primitive avian erythrocytes, but are the only α-globin genes expressed in definitive red cells. Interestingly, both types of red cells contain αA- and αD-globin in a ratio of approximately 3:1 (43). Our interest in the chicken adult α-globin genes derived from their somewhat unusual promoter structures (41). Both promoters lack any obvious CCAAT sequences which are typically found in other active globin genes (44), and little is known about the mechanism of their coordinate expression throughout development.

This paper describes a system in which the constitutive expression of an exogenous chicken αA-globin gene and *in vitro*-mutated constructs thereof can be monitored in analogy with studies of human globin gene expression in HeLa and COS cells. The αD-globin gene, however, is not expressed in this or any other tissue culture system that we have tested. Interestingly, when a DNA fragment containing both the αA- and αD-globin genes (in their normal arrangement), is transfected, αA-globin gene expression is substantially reduced and αD expression remains undetectable. This suggests the presence of a negative regulatory element which represses αA-globin gene expression in non-erythroid cells.

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MATERIALS AND METHODS

QT6 Cell Culture and Transfection

The QT6 Japanese quail cell line (45) was grown in Dulbecco's modified Eagle medium (Gibco Laboratories), supplemented with 4% fetal bovine serum, 1% chicken serum, 1% dimethyl sulfoxide (Fisher Scientific), and the antibiotics, penicillin and streptomycin (50 U/ml each). Stable transfections were performed using the calcium phosphate procedure (46). 5×10^5 cells were transfected with test plasmid DNA (20μg) and pSV2neo (46) plasmid DNA at a 2:1 molar ratio. G418 antibiotic (Gibco Laboratories) was added at 1 mg/ml 48 hr after transfection was begun. G418-resistant colonies were observable within 7−14 days after transfections, and coalescence into mass cultures occurred within the subsequent 1−2 weeks.

RNA Isolation

RNA was isolated from transfected cells by the guanidinium isothiocyanate extraction and CsCl gradient centrifugation procedures (47). RNA levels were assayed 4−6 weeks after transfection, and total RNA was prepared from 2−6 mass cultures for each transfection. Each RNA preparation was from cells expanded from about 10^3 resistant colonies.

S1 Nuclease and RNase Protection Assays

S1 analysis was performed as previously described (48) using 660−1320 U/ml of S1 nuclease in a 25°C incubation for 15 minutes. The 5′ end-labeled DNA fragments used in the assay were the BamHI (−814) to NarI (+112) fragment of pαA-1.7 and the 710 base pair (bp) StuI fragment (−630 to +81) of pαD-4.3 for αA- and αD-globin RNAs, respectively. Numerical designations of restriction sites are given with the respective transcription initiation or cap sites designated as +1 (41).

RNA probes were prepared by in vitro transcription of pT7-1 (U.S. Biochemical Corp.) clones containing the 342 bp Sau3AI (−172 to +170) fragment of the αA-globin gene and the 329 bp MspI (−51 to +278) fragment of the αD-globin gene, respectively. In both cases the plasmid DNA was linearized with HindIII digestion just downstream of the cloned insert. Labeled RNA transcripts were prepared with T7 RNA polymerase according to the directions of the supplier (U.S. Biochemical Corp.), followed by hybridization to total RNA samples and RNase treatment as described by Melton et al. (32). Protected RNAs were run on 6% sequencing gels as used for S1 analysis.

Plasmid Constructions

Subclones of α-globin gene-containing DNAs are as shown in Fig. 1. Plasmids pBRα7-1.7 and pHRα5-4.3 (called pαD-4.3 here for simplicity) have been described (41). Plasmid pHRα5-2.9 (pαA-2.9) contains the αA-globin gene from the nearest upstream EcoRI site to the downstream HindIII site, and pRStα5-1.8 (pαD-1.8) contains the αD-globin gene from the nearest upstream StuI site to the 3′ EcoRI site. Plasmid pαD+A contains both adult α-globin genes on a 5.6 kb fragment from an EcoRV site upstream of the αD-globin gene to the HindIII site just downstream of the αA-globin gene. All of these fragments were cloned into the corresponding sites of pBR322 or pAT153 plasmid DNAs (47) with the exceptions of StuI and EcoRV blunt-ended fragments for which vector EcoRV and NruI sites were used, respectively. The αA-globin gene subclone designated pαA-1.7 is identical to pBRα7-1.7 except that its vector (pATdT) contains a deletion from nucleotide 401 to 1283 (relative to EcoRI) in order to make further constructions easier.

The control chicken histone H3.2 gene plasmid DNA, pH3-L3′S, was derived from an EcoRI fragment containing the H3.2 gene of p3dR1 (50) cloned into the pSplink vector (51). An EcoRI-permuted Rous sarcoma virus (RSV) long terminal repeat (LTR) was inserted into the EcoRI site 3′ to the gene in the same transcriptional orientation as the histone gene.

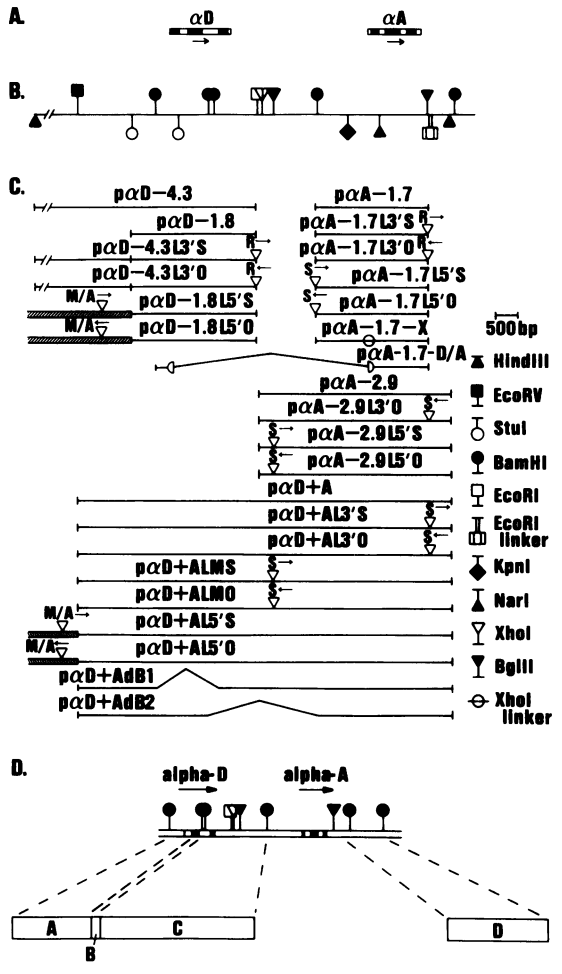

Figure 1. Restriction maps of clones used in transfections. A. The relative positions of the αA- and the αD-globin genes. Filled boxes represent protein coding sequences and open boxes represent intron and 3′ and 5′ untranslated sequences. Arrows denote the direction of transcription. B. The restriction map of the chromosomal DNA region containing the chicken adult α-globin genes. The EcoRI linker site arose only in a single λ recombinant during library construction but is shown since it is used in some subclones listed below. C. Regions of chromosomal DNA present in the various subclones. Triangles above the lines designate the position of inserted RSV LTR enhancers. Arrows above the LTR indicate the direction of RSV transcription in the provirus from which the LTR arose. In the diagram, R above the LTR indicates an LTR circularly permuted at its unique EcoRI site: such an LTR contains RSV sequences from −51 to +101 joined to −234 to −52 (15) in the direction of the arrow. S indicates an LTR permuted at the Sau3AI site which contains sequences −109 to +101 joined to −234 to −110 in the direction of the arrow. M/A indicates that portion of the LTR from MspI to AccI (+46 to +101 joined to −234 to −109 in the direction of the arrow), which contains the enhancer element (15). Deletions of sequences are indicated by joining the undeleted flanking regions with lines angled above the level of the subcloned DNA. The synthetic XhoI linker used in two constructions is as indicated (at a deletion site in pαA-D/A and the equivalent replacement in pαA-X). D. 5′- and 3′- flanking region BamHI fragments of the αA-globin gene, which were inserted into the BamHI and BglII sites positioned upstream (U) or downstream (D), respectively, to the αA gene in pαA-1.7 (see Fig. 8).

Subcloning of the RSV LTR into plasmids containing the α-globin genes was performed using permuted forms of the LTR, obtained by either EcoRI or Sau3AI digestion of two tandem LTRs followed by insertion at EcoRI, BamHI, or BglII sites, as appropriate. Regulatory regions within the 358 bp RSV LTR include both enhancer and promoter elements (52). In the permuted forms that were employed for subcloning purposes, the enhancer portion of the U3 region remained uninterrupted in order to maintain its potential effect on the α-globin genes, but the normal LTR promoter structure was either disrupted or deleted.

Miscellaneous

DNA sequence analysis of the chicken αA- and αD-globin genes has been described (41). Additional sequencing was performed by the method of Maxam and Gilbert (53) with modifications of Smith and Calvo, as reported (54). Plasmid DNA preparations, Southern blot analysis, DNA labeling, restriction enzyme digestion and bacterial strains were as described previously (41,42,51).

RESULTS

Characterization of constitutive globin gene expression in stably-transfected QT6 cells

Various adult chicken α-globin gene clones were initially transfected into mammalian cell lines (HeLa, COS7 and 293 cells) that have been shown to constitutively express several exogenous mammalian globin genes (9). In no case did we observe measurable expression of either chicken adult α-globin gene in stable or transient transfections of these mammalian cell lines (48 and results not shown). Apparently, transcription of chicken α-globin genes, unlike that of chicken vimentin (54, 55) or histone genes (S. Y. Son and J.B.D., unpublished results), requires one or more activities that are lacking in these mammalian cell expression systems.

When the chicken αA-globin gene (on pαA-1.7, Fig. 1) is stably co-transfected (with pSV2neo) into Japanese quail QT6 cells (45), an exogenous αA-globin transcript can clearly be detected (Fig. 2, lane 6). The band observed corresponds to RNA which starts at the normal cap site of the chicken αA-globin gene (41), so it appears that the initiation of transcription is normal in these cells. No transcription of endogenous globin genes in QT6 cells (normal or mock-transfected) is observed (Fig. 2, lane 7). (The endogenous quail αA-globin mRNA, if present, would be expected to cross-hybridize to the chicken probes up to approximately the translation initiation site (+37) producing a band about 35−40 nucleotides smaller than the exogenous gene band.)

QT6 cells are transformed fibroblasts (45), and thus it seemed likely that the level of exogenous αA-globin gene transcription would be increased by inserting an enhancer sequence known to function in fibroblasts into the αA-globin gene plasmid DNA. Fig. 2 (lanes 2 and 3) shows that the presence of a permuted RSV LTR enhancer 3′ to the αA-globin gene increased expression from the αA-globin promoter by about 100-fold. (The band in lane 5 resulting from loading 1/10 the normal assay sample following transfection with the LTR is about 10 times as intense as that in lane 6, resulting from loading all the sample after transfection without an LTR.) In this case, the permuted LTR was inserted at an EcoRI site about 900 bp 3′ to the αA-globin cap site (Fig. 1). In agreement with the definition of an enhancer sequence, comparable levels of expression were observed in cells transfected with clones containing the 3′ LTR in the same (Fig. 2, lanes 9 and 11) or opposite (Fig. 2, lanes 8 and 10) orientation with respect to the direction of gene transcription. The levels of expression from the clones with an LTR 3′ to the αA-globin gene were approximately two-fold greater than in clones containing LTRs 5′ to the gene (BamH

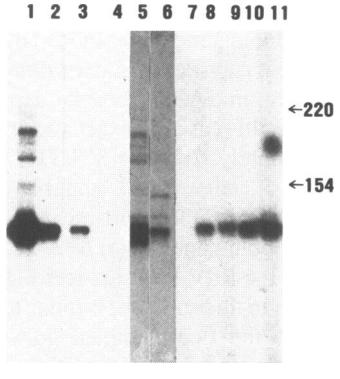

Figure 2. Comparison of the expression levels of the αA-globin gene with and without an RSV LTR in stably transfected QT6 cells. The RSV LTRs are positioned 3′- and 5′- to the gene with the LTR promoter in the same (S) or opposite (O) transcriptional orientation. RNA samples prepared from pooled stable transfectants were assayed by S1 analysis with a 5′ end-labeled αA-globin gene probe (−798 to +120, see Materials and Methods). S1 nuclease levels used were 660 U/ml in lanes 1−6 and 1320 U/ml in lanes 7−11. Equivalent results were obtained with either S1 level. RNAs used were: lane 1, total anemic chicken reticulocyte RNA; and total RNA from cells transfected with: lane 2, pαA-1.7L3′S; lane 3, pαA-1.7L3′S but with only 1/10 of the sample loaded; and lane 4, pαA-1.7. Lanes 5 and 6 are 10-fold longer exposures of lanes 3 and 4, respectively. Other RNAs used were: lane 7, total QT6 RNA; and RNA from cells transfected with: lane 8, pαA-1.7L5′0; lane 9, pαA-1.7L5′S; lane 10, pαA-1.7L3′0; and lane 11, pαA-1.7L3′S. With the exception of the reticulocyte RNA sample for which 10 μg of RNA was used, all assays were performed using 50 μg of RNA.

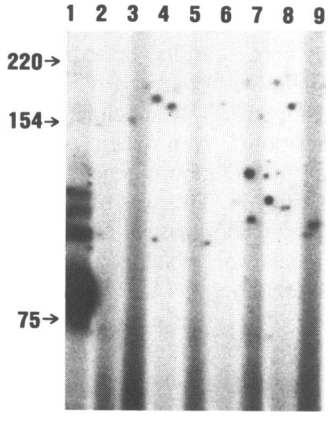

Figure 3. Absence of αD-globin gene transcripts in transfected QT6 cells. RNA was prepared from stably-transfected QT6 cells and used for S1 analysis with an end-labeled DNA fragment extending from −630 to +81 of the αD-globin gene (Materials and Methods). The film was overexposed to detect any possible transcripts in lanes 3−8. RNAs used were prepared from undifferentiated HD3 cells, lane 1; QT6 cells, lane 2; and QT6 cells transfected with: pαD-4.3, lane 3; pαD-4.3L3′S, lane 4; pαD-4.3L3′0, lane 5; pαD-1.8L5′S, lane 6; pαD-1.8L5′0, lane 7; pαD+A, lane 8; and pαD+AL3′S, lane 9. (RNA from cells transfected with pαD-1.8 lacking an LTR was not tested in this experiment but also showed no measurable expression of the αD gene in other experiments.)

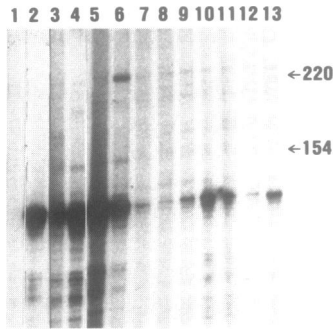

Figure 4. Reduced αA-globin expression levels in QT6 cells transfected with DNAs containing both αA and αD genes. Transfected plasmid DNAs included pαA-1.7 containing only the αA gene and flanking regions, and pαD+A containing both the αA- and αD-globin genes, with or without the RSV LTR (L) in the same (S) or opposite (O) transcriptional orientation with respect to the gene(s), and at the 5′- and 3′- ends of the gene(s). In the case of the larger clone, an LTR was also placed in the intergenic region approximately midway between the two genes (M). RNA samples (50 μg) were hybridized with an antisense RNA probe transcribed from the first exon region of the αA-globin gene, followed by RNase T1 and A digestions. RNAs assayed were QT6 cell RNA, lane 1; anemic hen reticulocyte RNA, lane 2; and RNA from QT6 cells transfected with: pαA-1.7, lane 3; pαA-1.7L3′0, lane 4; pαD-2.9L3′0, lane 5; pαD-2.9L5′S, lane 6; pαD+A, lane 7; pαD+AL3′S, lane 8; pαD+AL3′0, lane 9; pαD+A-LMS, lane 10; pαD+A-LMO, lane 11; pαD+A-L5′S, lane 12; and pαD+A-L5′0, lane 13. Bands in lanes 2, 4, 5 and 6 were overexposed to see those present in lanes 7 to 13.

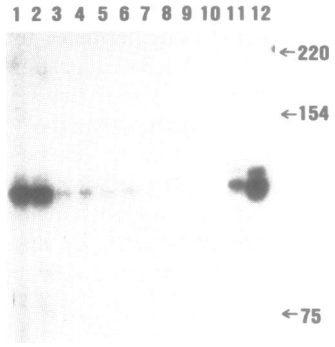

Figure 5. αA-globin gene transcript levels in cells stably co-transfected with αA- and αD-globin DNAs. The plasmid pαA-1.7L3′S was transfected in the presence and absence of unlinked αD-globin gene DNAs, or a chicken H3.3 histone gene plasmid as a control for co-transfecting with any DNA chicken clone. RNA samples (50 μg) were assayed for αA transcript levels as in Figure 2 with 660 U/ml (even-numbered lanes) or 1320 U/ml (odd-numbered lanes) of S1 nuclease. RNAs were from QT6 cells with: pαA-1.7L3′S, transfected singly, lanes 1 and 2 and pαA-1.7L3′S cotransfected with pαD-4.3, lanes 3 and 4; with pαD-4.3L3′S, lanes 5 and 6; with pαD-1.8, lanes 7 and 8; with pαD-1.8L3′S, lanes 9 and 10; or with pH3.3-L3′S, lanes 11 and 12.

I site at −814; Fig. 2, lanes 8 and 9). The two-fold difference is probably not significant and may relate to the fact that different permutations of the LTR sequence were used (Fig. 1). Note that the enhancing effect specifically increases the level of RNA initiated at the αA-globin gene cap site. We have not attempted to measure the level of transcripts initiating within the LTR itself, but the fact that the LTRs used in these constructions have their 5′ promoter-flanking sequences rearranged or deleted and the fact that the enhancement is relatively position- and orientation-independent argue that this effect is not directly related to the promoter function of the LTR sequences.

In contrast to the above results, no expression of the closely linked αD-globin gene was observed when either the pαD-4.3 or the smaller pαD-1.8 (Fig. 1) subclone was used to transfect QT6 cells. Furthermore, even when permuted LTRs were inserted 5′ or 3′ to the αD-globin gene, that gene was inactive in transfected cells (Fig. 3). This was true using either S1 assays (Fig. 3) or RNase protection assays (not shown), even though easily detectable signals were obtained in both cases using either total anemic hen reticulocyte RNA or undifferentiated HD3 (56) total RNA. The HD3 cell line is a *ts* AEV-transformed cell line which consists predominantly of erythroblasts but still expresses low levels of globin mRNAs (and other red cell mRNAs such as carbonic anhydrase II, 57) in the undifferentiated state used here (41). Generally, undifferentiated HD3 cells express about 2% of the levels of globin and carbonic anhydrase II mRNAs as do anemic hen reticulocytes (57). Since it appears that the αD-globin RNA assays are of comparable sensitivity to those used successfully for αA-globin RNA, we estimate that constitutive expression of the αA-globin gene in QT6 cells is at least 10−20 times higher than that of the αD-globin gene in the absence of an RSV LTR enhancer and over 1,000 times higher when both are in the presence of a linked LTR enhancer sequence.

αA-Globin Expression from Linked Gene Clones

Since the chicken αA- and αD-globin genes are closely linked in chromosomal DNA and coordinately expressed *in vivo*, the large difference in their expression levels in transfected QT6 cells was unexpected. Therefore, a larger clone, pαD+A (Fig. 1), was prepared that contained both genes linked just as they are in the chicken genome (42). Following stable transfection of this clone into QT6 cells, both RNase protection (Fig. 4) and S1 analysis (not shown) demonstrated a low level of αA-globin gene expression, approximately 1/10 of that observed from transfection of the pαA-1.7 clone described previously (Fig. 4, compare lane 7 to lane 3). Furthermore, insertion of the RSV LTR enhancer directly 3′ to the αA-globin gene in this clone (BglII site at +850 relative to the αA-globin cap site) in either orientation gives only a slight increase, if any, in αA-globin gene expression (Fig. 4, lanes 8 and 9). Since in this case the exact site and configuration of the LTR differed slightly from the 3′ LTRs used previously in pαA-1.7L3′S (Fig. 2, lanes 2, 10, and 11; Fig. 4, lane 4), we also tested the effect of LTR insertion at the identical BglII site in the larger αA-globin gene clone, pαA-2.9 (Fig. 1). Lane 5 of Fig. 4 shows that the RSV LTR induces high levels of αA-globin gene expression in this subclone similar to induction seen by LTR insertion into the EcoRI site of pαA-1.7. Thus, the presence of the upstream region of DNA which contains the αD-globin gene (in pαD+A) appears to mask the enhancing effects of LTR insertion which are seen in pαA-2.9 (Fig. 4, compare lanes 5 and 9). When the permuted LTR was located in the BglII site upstream of the αA-globin gene in pαD+A (approximately 1.5 kb 5′ to the cap site, Fig. 1), a slight (2−3-fold) but measurable enhancement was observed (Fig. 4, lanes 10 and 11). However, the level of enhancement in this clone containing both the αA- and αD-globin genes was considerably less than that exhibited by the same LTR placement in the pαD-2.9 clone (Fig. 4, compare lanes 6 and 10). No αD-globin gene expression was observed in transfections with all seven of the pαD+A-derived clones (results shown only for two of them, see Fig. 3, lanes 8 and 9).

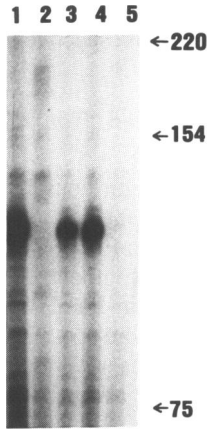

Figure 6. S1 analysis of hybrid globin gene expression in stably transfected QT6 cells. QT6 cells were stably transfected with the clones pαA-1.7; pαA-X, an αA-globin plasmid with the 8 bp sequence at bp −15 to −7 replaced by an XhoI linker; and pαA-D/A, a hybrid plasmid clone consisting of the αD-globin promoter and the αA-globin gene body, joined at an XhoI linker at bp −15 to −7. S1 (1320 U/ml) analysis was as described in Fig. 2. RNA was extracted from anemic hen reticulocytes, lane 1; QT6 cells, lane 2; or from QT6 cells transfected with pαA-1.7, lane 3; pαA-X, lane 4; and pαA-D/A, lane 5. 50 μg of each RNA was used except for total reticulocyte RNA of which 10 μg were used.

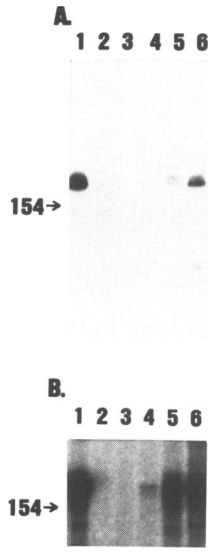

Figure 7. αA-globin gene expression in QT6 cells transfected with deleted derivatives of pαD+A. A. RNase protection analysis as in Fig. 4 of RNA (50 μg) isolated from undifferentiated HD3 cells, lane 1; QT6 cells, lane 2; and QT6 cells stably transfected with: pαD+A, lane 3; pαD+AL3′S, lane 4; pαD+AdB1, lane 5; or pαD+AdB2, lane 6. B. Same as A except exposed for 5 times longer. pαD+AdB1 deletes two BamHI fragments (807 bp and 80 bp) 5′ to and within the αD-globin gene; pαD+AdB2 deletes a BamHI fragment of 1.56 kb from intron 2 of the αD gene to nucleotide −814, relative to the αA-globin gene (see Fig. 1).

The constitutive expression of the αA-globin gene in QT6 cells was therefore in some way repressed when linked in *cis* to the αD-globin gene region (in the normal chicken chromosomal DNA arrangement). Furthermore, this repression appeared, for the most part, to be dominant to the enhancing effect of a linked RSV LTR, even when the LTR was on the side of the gene opposite from the repressing DNA. In order to verify that this was an effect on gene expression and not on co-transfection frequency, Southern blots of DNA isolated from pooled transfectants were performed. Hybridization with a portion of the transfected αA-globin gene DNA shows that co-transfection with pαD+A results in integration of the exogenous chicken sequences into the genome of the selected QT6 cells just as does co-transfection with pαA-1.7 (results not shown). No consistent differences were observed in the copy numbers of integrated transfecting DNAs between the pαA-1.7L3′S plasmid, which lacks the αD-globin gene region, and those plasmids such as pαD+AL3′S which contain the αD-globin gene. Furthermore, pooled stable transfectants were used to eliminate any bias due to effects of specific sites of integration. Thus transfection efficiency does not appear to account for the decrease in αA-globin gene transcript level when linked to the αD region. The studies described below also argue against the reduction in αA-globin gene expression being due to altered transfectability.

αA-Globin Gene Expression in αA- and αD-Globin Gene Co-Transfections

Since the expression of the αA-globin gene in QT6 cells was reduced when linked to the αD-globin region DNA, we also wished to test the result of co-transfecting the αA- and αD-globin gene DNAs on separate plasmids. As a control, the αA-globin gene plasmid was also co-transfected with a plasmid containing the chicken H3.2 histone gene (pH3-L3′S, Materials and Methods). Of course, all co-transfections also contained the pSV2neo plasmid used for G418 selection of stable transfectants. Expression of the αA-globin gene DNA with a 3′ permuted LTR enhancer sequence (pαA-1.7L3′S) was comparable in cells transfected with the αA-globin gene alone or co-transfected with the histone H3.2 gene DNA containing a 3′-LTR (Fig. 5, compare lanes 1 and 2 to lanes 11 and 12). In similar co-transfections with αD-globin gene-containing plasmids (with or without an LTR on the αD-globin plasmid), the levels of expression of the αA-globin gene dropped to approximately 10% or less of the singly transfected αA-globin gene level (Fig. 5, compare lanes 1, 2 to lanes 3−10). No αD-globin expression was detectable in cotransfections involving these DNAs (data not shown). While this might suggest that the repression of αA-globin expression exerted by the αD-globin gene region also occurs in *trans*, this is not necessarily the case. The transfected DNAs are expected to form a mixed tandem multimer on integration (59), and thus at least some of the transfected αA-globin genes are likely to be linked to nearby αD-globin gene region DNA. Southern blotting of pooled co-transfectant DNAs is in agreement with this possibility (not shown).

αA-Globin Gene Expression in Transfections of Hybrid Genes

A hybrid α-globin gene was constructed which contained the αD-globin gene promoter linked to the cap site and body of the αA-globin gene. This clone was termed pαA-D/A. Since it was constructed by joining deletion clones with an 8 bp XhoI linker at bp −15 to −7 between the TATA sequence and the cap site (+1), a control clone, pαA-X, was constructed which is identical to the pαA-1.7 used previously except that bp −15 to −7 are replaced by the XhoI linker. Fig. 6 (lanes 3 and 4) shows that, as expected, the mere insertion of the XhoI linker into pαA-X had no effect on transcription level in transfected QT6 cells. However, when the αD-globin gene promoter was exchanged for the αA-globin gene promoter, expression was essentially

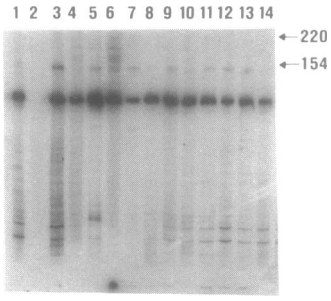

Figure 8. αA-globin gene expression in QT6 cells transfected with pαA-1.7L3'S subclones containing 5'- and 3'-flanking region insertions. Insertion sites are upstream (U) at the BamHI site at −814 and downstream (D) at the BglII site, +859, with BamHI fragments shown in Fig. 1D subcloned in the same (S) or opposite (O) orientation as compared to that in the normal α-globin gene locus. RNAse protection assays, using the αA-globin riboprobe described in Materials and Methods, were performed using 50 μg of total RNA from QT6 cells transfected with; pαA-1.7 (lane 1); pSV2Neo (lane 2); pαA-1.7C-DS (lane 3); pαA-1.7C-US (lane 4); pαA-1.7C-DP (lane 5); pαA-1.7C-UO (lane 6); pαA-1.7A-DS (lane 7); pαA-1.7A-US (lane 8); pαA-1.7A-DO (lane 9); pαA-1.7A-UO (lane 10); pαA-1.7B-DS (lane 11); pαA-1.7B-US (lane 12); pαA-1.7B-DO (lane 13); pαA-1.7B-UO (lane 14).

completely abolished (Fig. 6, lane 5). This implies a fundamental difference in regulation between the two chicken adult α-globin gene promoters when present in QT6 cells.

When the reciprocal recombinant was constructed, i.e., by attaching the αA-globin gene promoter to an αD-globin gene body, no measurable αD-globin gene expression was observed (data not shown). This implies that the promoter of the αA-globin gene, but not that of the αD-globin gene, is active in the QT6 system and suggests that sequences in the neighborhood of the αD-globin gene lower expression from an otherwise active αA promoter.

Expression from Clones Containing Deletions of Intergenic and αD Gene Sequences

The results described above indicate that sequences present in the 5.6 kb insert of pαD+A but absent in pαA-1.7 (Fig. 1) reduce αA-globin gene expression in transfected QT6 cells. In order to localize this effect, two deletion derivatives of pαD+A were prepared and assayed. Fig. 7A (lane 3) shows that, as described above, the pαD+A clone gives rise to very little αA-globin gene expression in QT6 cells. Insertion of an RSV LTR enhancer 3' to the gene still gives rise to only low levels of αA-globin expression (Fig. 7B, lane 4). Two BamH I fragments (807 and 80 bp) were deleted from the αD-globin gene region of pαD+A to form pαD+AdB1 (Fig. 1). The region deleted extends from −251 to +637 with respect to the αD-globin gene (17) and spans the αD-globin promoter region through most of intron 2. When pαD+AdB1 was used to transfect QT6 cells (Fig. 7A and B, lane 5), αA-globin gene expression increased about 20-fold with respect to pαD+A. A second deleted subclone, pαD+AdB2 (Fig. 1), lacks a 1.56 kb BamHI fragment, with the deleted region extending from +638 to +2201 with respect to the αD-globin cap site. (This deletion ends at position −814 with respect to the αA-globin cap site.) Expression of the αA-globin gene on pαD+AdB2 (Fig. 7A and B, lane 6) is nearly 100-fold greater than that on pαD+A. Thus, deletion of either of two different portions of the αD-globin gene-containing region results in significantly increased expression from the αA-globin gene promoter, even though the deletion endpoints are 2.4 or 0.8 kb, respectively, upstream of the αA-globin gene cap site.

αA-Globin Gene Expression from Subclones Containing Flanking Region Insertions

Regions implicated by deletion constructs in the inhibition of expression were tested for orientation- and position-independence of their effects. The upstream (αD gene-containing) region was divided into various BamHI fragments as shown in Fig. 1D. We also included the downstream BamHI fragment (D in Fig. 1) shown to contain the erythroid-specific enhancer region (62). Various fragments were inserted in both orientations into upstream (BamHI, −814) or downstream (Bgl II, + 850) sites (Fig. 1D). Results showed that αA-globin gene expression remained essentially unchanged when monitored in all subclones (Fig. 8, lanes 1−12). Comparisons revealed less than five-fold differences in expression between the αA gene wild-type and modified clones. These results are in contrast with the reduction in αA expression in the presence of an intact αD-globin gene (unlinked in separate clones or tandemly linked in the double clone).

αD- to αA-Globin Intergenic Region Nucleotide Sequence

In the chicken genome, the α-globin gene family is comprised of three genes arranged in a 5'- to 3'-order: π--αD--αA. The individual nucleotide sequences of these three genes have been reported (41,63). The complete sequence of the adult α-globin gene region from 521 nucleotides upstream of αD to 170 nucleotides downstream of αA is shown in Figure 9. This includes regions deleted or rearranged in constructs described in this paper. Analysis of the sequence data revealed no further consensus elements beyond those already described (40,41).

DISCUSSION

We began using the transfected QT6 cell system in order to study constitutive regulatory sequences in and around the chicken globin genes. The behavior of the chicken αA-globin gene in this system is intermediate to that of the human α- and β-globin genes in HeLa cells (6,9). Like that of the human α-globin gene, αA-globin gene expression in the absence of an enhancer is clearly detectable, but the 100-fold level of enhancement by the RSV LTR enhancer is similar to that of the β-globin gene by the SV40 enhancer as opposed to the 5−10-fold enhancement of the human α-globin gene. These quantitative differences are not surprising, given the differences in expression systems being employed. In this regard, there must be at least one avian-specific factor in the QT6 cells that is absent in mammalian globin gene expression systems, since transfected chicken αA-globin genes are not expressed in any mammalian cell system we have tested, either by transient or stable transfection assays.

It should be pointed out that all the results presented employed stable transfection procedures. Therefore, the exogenous DNAs were integrated into QT6 quail chromosomal DNA and presumably organized into normal nucleosomal structures. Over a thousand transfectants were used for each assay in order to average out the effects of integration position on exogenous gene expression. Of course, in all cases the exogenous DNA probably exists as a multimeric cointegrate (60). Chromosomal blotting of pooled transfectant DNAs indicated that exogenous globin gene

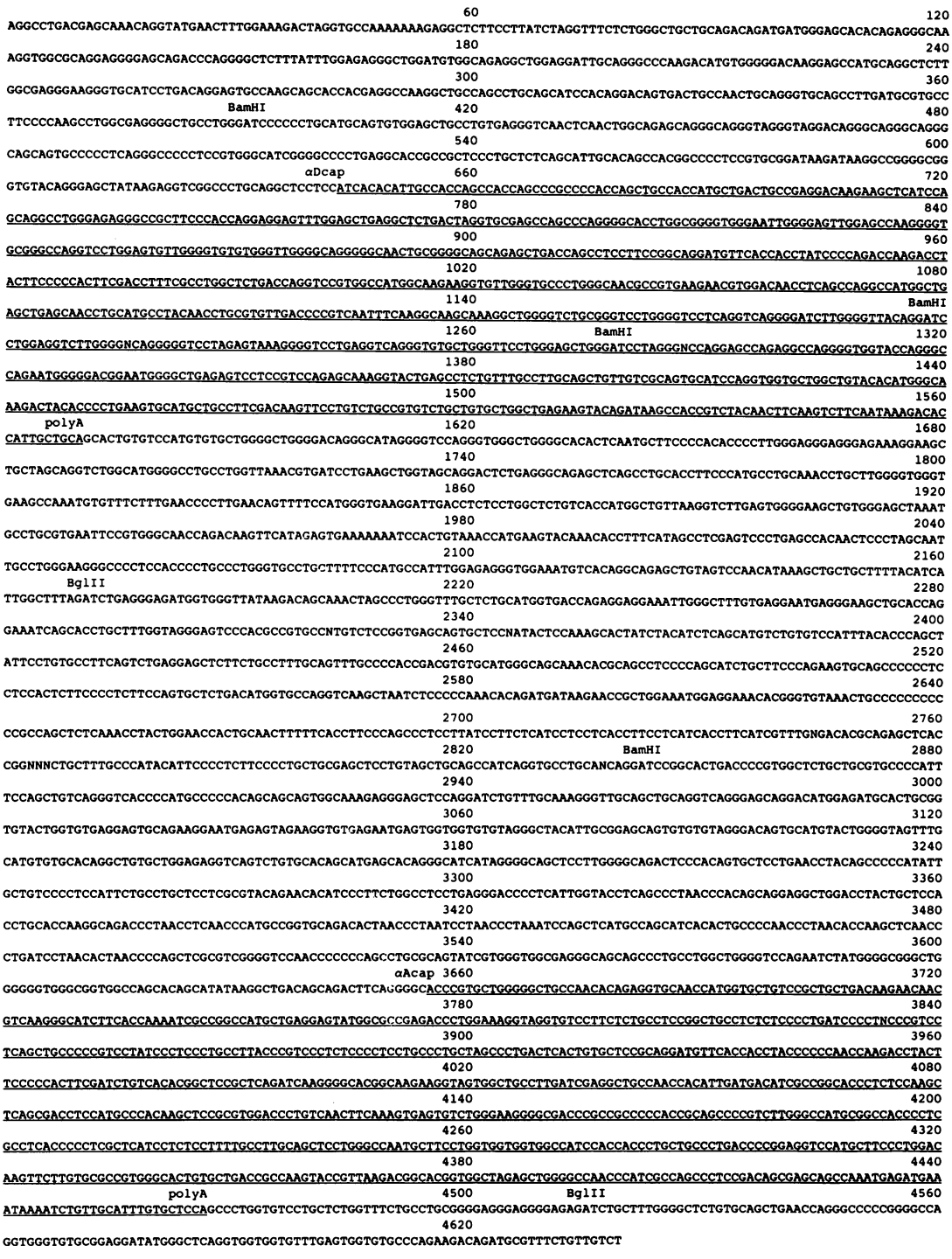

Figure 9. DNA nucleotide sequence of the region comprising the αD-globin gene, the αD- to αA-globin intergenic region, and the αA-globin gene. Previously published sequences include nucleotides 337 to 1804 and 3355 to 4618. Transcribed regions of αD and αA genes are underlined. Cap and putative poly (A) sites are as follows: αD cap, 642, and poly (A), 1573; αA cap, 3655, and poly (A), 4466. This sequence is Accession Number X59989 in the EMBL Data Library.

DNA sequences were present in copy numbers of about two to five on average. Copy numbers of the selected pSV2neo plasmid were similar.

The considerable difference between αA- and αD-globin gene expression in QT6 cells was unexpected since, although the two genes have evolved separately for a long period, their two promoters are fairly similar in general appearance (41). It might be argued that the lack of measurable αD-globin gene expression is more 'natural' since QT6 cells are fibroblast in nature and don't express their endogenous αglobin genes. However, transfected genes often are expressed where their endogenous counterparts are not (1,5,9). The αD-globin gene is also very poorly expressed in transient QT6 transfections (our unpublished results) which, along with the results described above, suggests that this trait is a particular aspect of the αD-globin gene. Whether the transfected αD-globin genes are blocked by a mechanism that

acts normally in vivo to prevent their expression in non-erythroid cells and, if so, how this mechanism is relieved during erythropoiesis remain to be determined.

Our data are consistent with the presence of a repressing sequence in the αD-globin gene coding region which acts on the αA-globin gene promoter. In some aspects the effects we observed are similar to those observed by Choi and Engel in the chicken β-globin gene cluster (13). These investigators identified a locus in the β-globin gene promoter which blocks expression of the linked ε-globin gene by competition for regulatory DNA binding proteins. However, in that case the β-globin gene transcription and ε-globin gene suppression occurred simultaneously, whereas in our system αD-globin gene expression was not observed at the time of the αA-globin gene inhibition. Moreover, in vivo, β- and ε-globin gene activities are mutually exclusive, while αD- and αA-globin genes are simultaneously expressed. In another report of a silencer effect between two globin genes (one in which gene expression is not mutually exclusive), Atweh et al. reported inhibition of human β-globin gene expression in the presence of a linked α-globin gene (32).

In the present experiments, inhibition of αA-globin gene expression was observed in the presence of separate or linked αD clones with 5′- or 3′- flanking sequences of various lengths. Sequences that were in common among the inhibitory αD gene constructs were limited to the αD-globin coding region and an additional 300 bp 3′ to the gene. However, in transfections of constructs in which these common sequences were rearranged either 5′ or 3′ to the expressed αA gene, an effect upon transcription was not observed, nor was an effect observed with clones containing inserts from other 5′ and 3′ regions of the α-globin gene locus (Fig. 8). Inhibition of the αA-globin gene by the presence of the αD-globin gene region may be dependent on a chromatin structure that is disturbed by insertion or deletion of ancillary fragments on either side of the αA-globin gene. In other words, expression of the αA gene may depend on the proper positioning of one or more as yet undefined *cis*-acting sequences (e.g., as mediated by nucleosome phasing). In this model, the effect, for example, of a given deletion relates not to the region deleted but to a change in the relative position of elements that remain in the construct. When constructs such as those described in Fig. 1 are tested for αA-globin gene expression in a transient transfection system, an inhibitory effect of the αD gene region is observed, but the effect is considerably less pronounced (data not shown). This is consistent with the possibility that normal chromatin structure must be established in the region of the exogenous DNA to observe maximal inhibition. A second possibility is that the effective site(s) within the αD-globin gene or its immediately adjacent sequences has been disrupted in the various insertions. Our results with the two large deletion clones are consistent with this possibility. Deletion of fragments A and B together or C alone in Fig. 1D reverses the inhibitory effect, so, if this second explanation is correct, the region around the B/C border in the second intron of the αD gene would be implicated. Alternatively, two or more sequence elements which flank the B/C junction may be involved which must remain in a specific orientation with respect to one another to exert their effect.

A wide variety of studies indicate that developmental gene regulation often involves an interplay between several tissue-specific and constitutive factors and their binding sites. For example, a combinatorial role of constitutive and tissue-specific factors, and one involving the unusual possibility of developmental regulation of a constitutive factor, has been cited for chicken β-globin gene expression (64). The *in vivo* relevance of observations reported herein, concerning the regulation of αA- and αD-globin gene constitutive expression and interactions between the two distantly related but coordinately expressed gene sequences, remains to be resolved.

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