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Light chain gene conversion continues at high rate in an ALV-induced cell line

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We have analyzed immunoglobulin light chain sequences from avian leukosis virus (ALV) induced bursal and metastatic tumors and from cell lines derived from these tumors. Sequence data presented demonstrate that ALV-induced tumors and one cell line (DT40) derived therefrom continue to diversify their light chain genes outside of the bursal environment. Diversification within these tumor cells seems to occur by gene conversion events comparable with those observed in bursal B cells. Sequence analysis of spontaneously arising surface immunoglobulin negative subclones of the DT40 cell line revealed frameshifts within the rearranged light chain genes which most likely resulted from non-functional recombination events. Superimposed gene conversion events can repair these frameshifts leading to re-expression of surface immunoglobulin.

Key words: ALV virus/B cell lines/immunoglobulin genes/gene conversion

Introduction

Chicken B cell precursors rearrange their immunoglobulin genes before or during colonization of the bursa of Fabricius (Weill *et al.*, 1986; McCormack *et al.*, 1989). Since rearrangement is limited to a single functional V and J gene segment at the light chain locus it contributes little to the development of the chicken light chain immunoglobulin repertoire (Reynaud *et al.*, 1985). Subsequent B cell proliferation within bursal follicles is accompanied by diversification of the rearanged light chain genes through segmental gene conversion with a nearby group of pseudogenes serving as donors (Reynaud *et al.*, 1987; Thompson and Neiman, 1987). The rate of somatic gene conversion within bursal B cells has been estimated to be as high as one event per 10-20 cell divisions (Reynaud *et al.*, 1987). Since the boundaries of conversion events are variable and such events occur repeatedly in the bursa, diversification through gene conversion is very efficient and seems capable of creating the very large preimmune repertoire (Weill and Reynaud, 1987; Pink, 1986). Starting at about the time of hatching, B cells migrate from the bursa into peripheral organs where they install the adult B cell compartment. It is not known whether immunoglobulin gene diversification continues outside the bursal environment in the periphery.

The study of somatic diversification of chicken immunoglobulin genes can be aided by the analysis of lymphomas induced by the avian leukosis virus (ALV). Neonatal ALV infection of susceptible strains of chickens causes B cell lymphomas with a latency of several months. Appearing first in the bursa, these lymphomas later seed metastases to the liver, spleen and other visceral organs (Purchase and Burmeister, 1978). The initiation of tumor development is linked to proviral insertions near the c-*myc* locus and most tumors are clonally marked by a unique proviral integration site (Neel *et al.*, 1981).The finding of restriction site heterogeneities within rearranged VJ gene segments of ALV-induced tumors and a cell line (DT40) derived therefrom indicated that light chain gene diversification continues during tumor development (Thompson and Neiman, 1987; Thompson *et al.*, 1987).

The present study was undertaken to determine by sequence analysis the nature and rate of light chain gene diversification within ALV-induced tumor cells. Here we report evidence for on-going gene conversion in all *in vivo* tumors examined. But among several cell lines studied, only one showed intraclonal light chain gene heterogeneity. To characterize possible errors in the recombination mechanism we further analyzed surface immunoglobulin negative ($sIgM^-$) subclones derived from this cell line and their $sIgM^+$ revertants.

Results

Light chain diversification in ALV-induced tumors

Rearranged light chain VJ gene segments of a bursal tumor and a thymic metastasis from it were amplified by the polymerase chain reaction (PCR). Cells of the original bursal tumor and its thymic metastasis were determined by Southern blot analysis to be related to each other showing the presence of an identical proviral integration site near the *myc* locus (data not shown). Four sequences were determined for each tumor. Figure 1 shows these sequences aligned with the germline VJ sequence of the CB inbred chicken line. Any differences that can be accounted for by sequences of the pseudogene pool of that inbred strain (Reynaud *et al.*, 1987) are highlighted by boxes. The comparison shows that tumor sequences differ from each other by sequence motifs derived from the pseudogene pool. Since analysis of the proviral insertion sites demonstrate that these tumors originated from a single cell, the differences indicate that light chain diversification continues by gene conversion events after cell transformation.

Surprisingly, sequences derived from the bursal tumor do not appear to be more similar to those from its thymic metastasis than to sequences derived from other unrelated tumors (unpublished results), indicating that accumulating light chain gene modifications obscure their common origin. We believe this phenomenon is due to clonal dominance of a cell population within the bursal tumor that is no longer

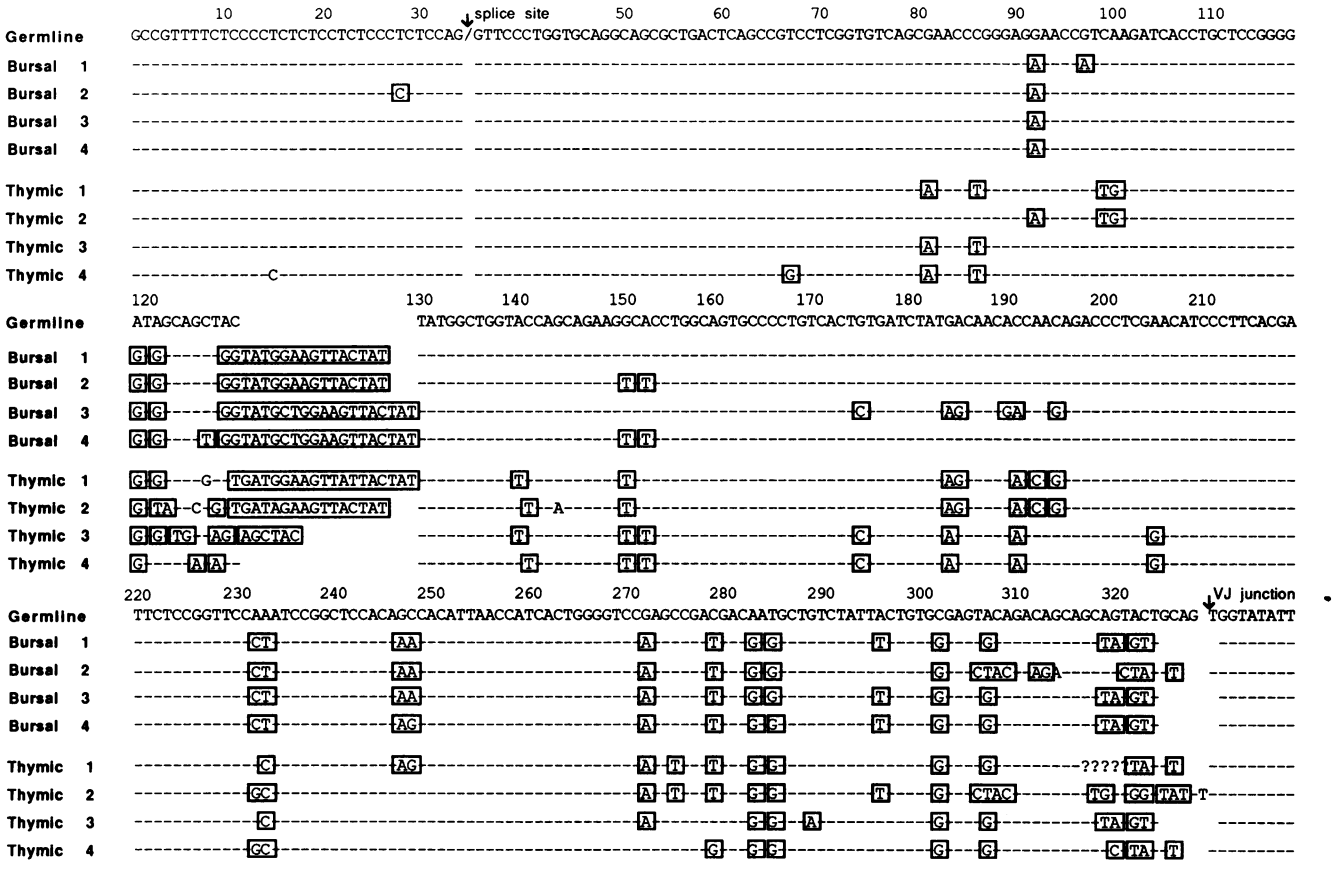

Fig. 1. Light chain gene sequence obtained from an ALV-induced bursal tumor and its thymic metastasis. All sequences were aligned with the germline VJ sequence of the CB chicken inbred line and any sequence differences which can be accounted for by sequences of the pseudogene pool of the CB chicken (Reynaud *et al.*, 1987) are highlighted by boxes. The question marks within the Thymic 1 sequence are inserted at positions where the sequence remained ambiguous due to compressions.

closely related to the cell clone which gave rise to the metastasis (Korczak *et al.*, 1988).

On-going diversification in a cell line

To address the question whether ALV-induced tumor cell lines continue diversification of their rearranged light chain genes we first determined multiple sequences from the cell line LCSS-RP9 (Okazaki *et al.*, 1980) as well as from sixteen newly established ALV-induced cell lines (D.Ewert *et al.*, manuscript in preparation). Only three of the new cell lines show sequence heterogeneity, and that at a low level. However, subclones of these cell lines yielded uniform sequences (data not shown). Gene conversion activity at the rearranged light chain locus may be too low to be detected in these cell lines by random sequencing, if it continues at all.

Heterogeneity of *Kpn*I restriction enzyme site within the rearranged V gene segment of a subclone of the ALV-induced DT40 cell line was reported as evidence that light chain diversification continued during cell culture (Thompson *et al.*, 1987). We therefore repeated the subcloning of the DT40 cell line. Four sequences of each of two subclones (SC1 and CL16) are shown in Figure 2. Although sequences of the same subclone have many modifications from the germline sequence in common, they clearly differ from each other by sequences present in the pseudogene pool. Notably, the recognition site of the *Kpn*I enzyme between bases 137 and 142 is modified in only three of the four sequences of the SC1 subclone. The CL16 subclone was obtained from a population enriched for $sIgM^-$ cells (see below), but was later shown to be predominantly $sIgM^+$ (data not shown). An unusual deletion of base pairs 183–198 in all four sequences of this subclone argues against the possibility that intraclonal sequence heterogeneity was due to mixed subcloning.

To estimate the minimal frequency of gene conversion events one may take the most common sequence of the SC1 and CL16 subclones as the hypothetical sequence of their progenitor cell at the start of the subcloning. Under this assumption the SC1.1 sequence has undergone a templated sequence modification at base pair 81, the SC1.2 sequence at base pairs 140 and 248, the SC1.3 sequence at base pairs 231–233, and the SC1.4 sequence at base pairs 81, 125 and 175 (Figure 2). Similarly, an average of one conversion event per sequence could account for the observed intraclonal sequence differences of subclone CL16. Since the doubling time of DT40 cells is ~12 h and DNA was isolated 3 weeks after subcloning, gene conversion events may occur at an average rate of one per 40 cell divisions.

Unusual sequence modifications

A few single base pair substitutions in the light chain gene sequences of the tumors and subclones of DT40 cannot be accounted for by sequences derived from the pseudogene pool (Figures 1 and 2). We cannot rule out the possibility that they are due to errors of the Taq polymerase during sequence amplification. However similar single base pair

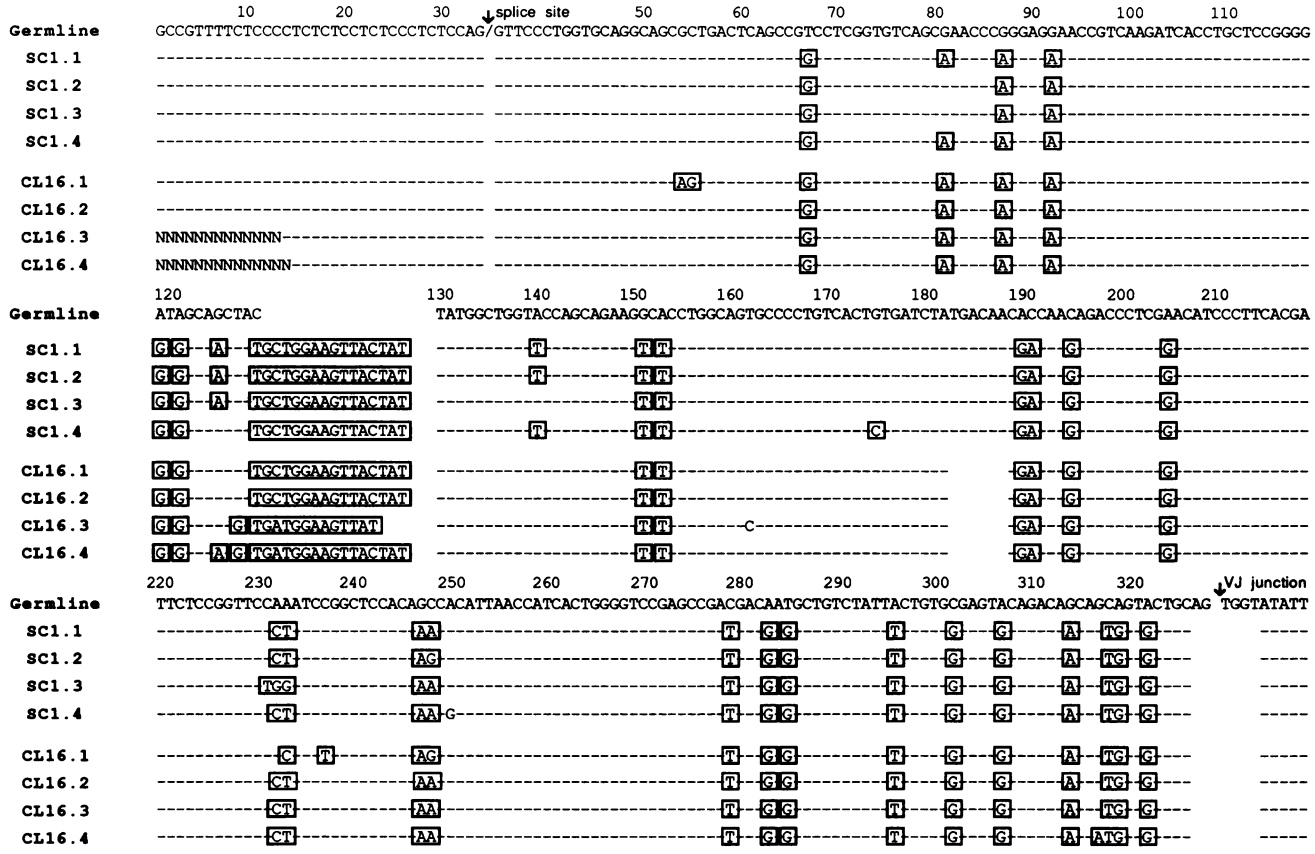

Fig. 2. Sequences of light chain genes from DT40 subclones SC1 and CL16. Sequences are aligned and differences are highlighted as in Figure 1. Undetermined bases at the 5′ end of the CL16.3 and CL16.4 sequences are indicated by the letter 'N'.

substitutions have been previously detected in sequences of bursal B cells (Reynaud *et al.*, 1987).

A number of other sequence modifications in the regions encoding complementarity determining region 1 (CDR1) and CDR3 can only be explained by known pseudogene sequences, if one postulates the occurrence of codon shifts. This is illustrated by the examples of Thymic 1 and CL16.3 sequences between base pairs 120 and 140 (Figure 3A and B). Conventional alignment of the V8 and V18 pseudogenes can result only in five codon insertions, whereas the Thymic 1 and CL16.3 sequences contain a six and four codon insertion, respectively. We argue that these results are due to unusual alignments of the pseudogene V18 sequence upon a sequence previously converted by pseudogene V8. Remarkably, termination of sequence transfer would have occurred in these cases within stretches of six and five homologous base pairs as indicated by arrows in Figure 3.

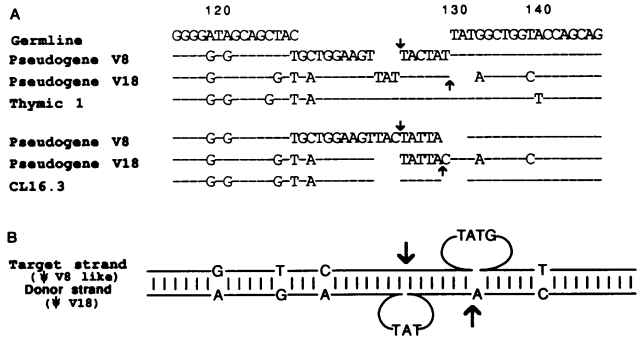

Fig. 3. (**A**) Proposed alignment of pseudogene V8 and V18 during gene conversion events that may have resulted in the Thymic 1 and CL16.3 sequences between base pairs 130 and 140. Arrows indicate the regions of the proposed shifted alignment. (**B**) Hypothetical heteroduplex intermediate of the conversion event that led to the Thymic 1 sequence. The extent of the heteroduplex formation remains speculative.

Lack of sIgM expression is reversible in DT40 cells

The on-going recombination during cell culture of the DT40 cell line encouraged us to search for potential errors of the conversion mechanism. As determined by surface staining with an anti-chicken IgM monoclonal antibody the DT40 bulk population does not contain a discrete $sIgM^-$ subpopulation (Figure 4a,b). However, the $sIgM^-$ cells become clearly visisble after two rounds of killing with M-1 monoclonal antibody (mAb) and complement (Figure 4c,d). $sIgM^-$ cells were further enriched by FACS sorting followed by subcloning. Three weeks after subcloning 17 of 20 subclones still contained a predominantly $sIgM^-$ population (the immunofluorescence histograms of one of these subclones (CL18) are shown in Figure 4e,f). The three other subclones, including CL16, stained predominantly $sIgM^+$. The presence of a small (0.5–5%) $sIgM^+$ subpopulation in 16 of the 17 $sIgM^-$ subclones suggested that most $sIgM^-$ cells can revert to $sIgM^+$ cells. This view was further corroborated by the finding that subclones of the clones CL18 and CL20 contained a small $sIgM^+$ subpopulation after three weeks growth (data not shown).

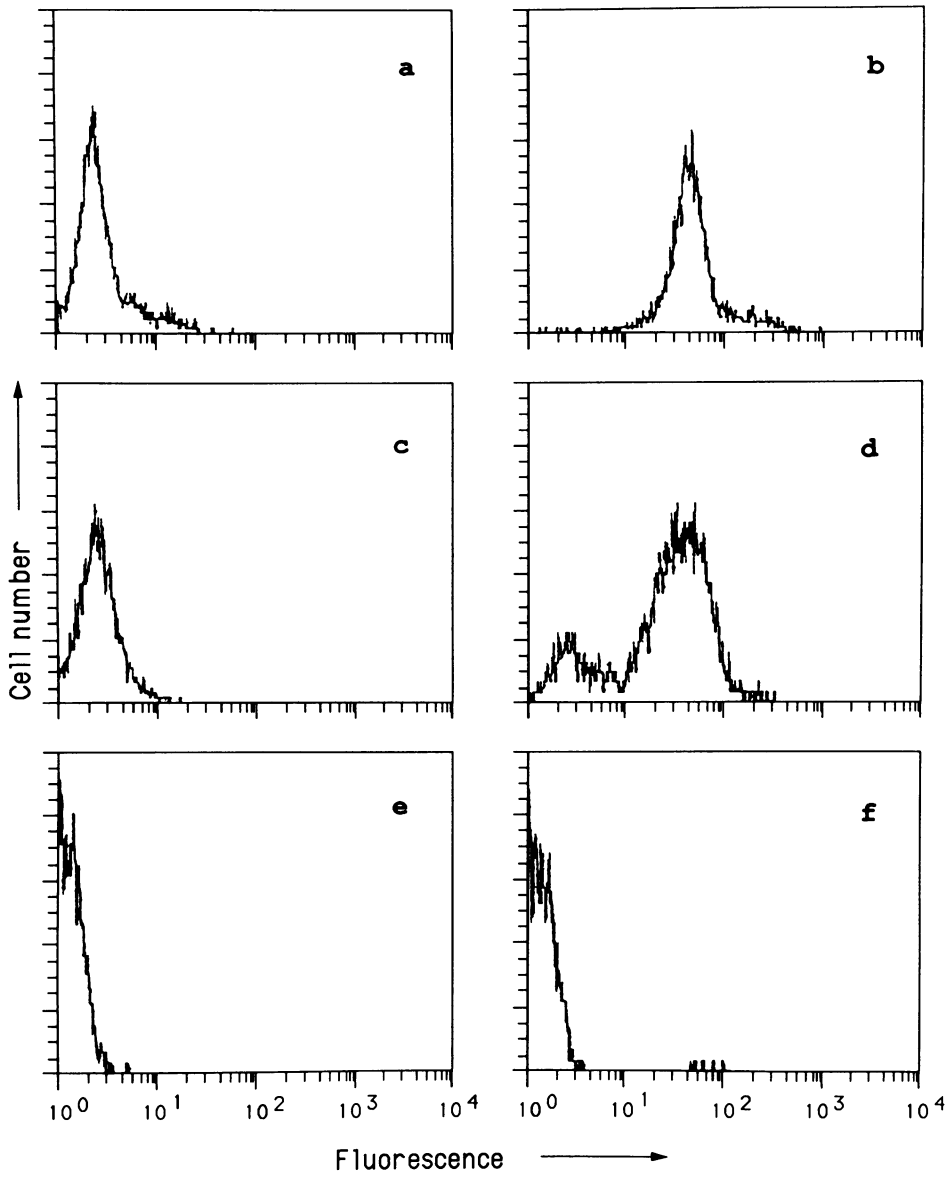

Fig. 4. Histograms of DT40 cell populations after surface staining with the anti-light chain mAb M-1. (**a,b**) DT40 bulk population. (**c,d**) DT40 cells after two cycles of enrichment for IgM^- cells. (**e,f**) subclone CL18. The histograms labeled (a), (c) and (e) represent negative control stainings.

Frameshift repair by conversion events

The light chain genes of all $sIgM^-$ subclones were amplified by PCR and sequenced. Since most bursal cells as well as ALV-induced tumor cells contain only one functionally rearranged light chain gene, the other allele being in germline configuration (Reynaud *et al.*, 1985; McCormack *et al.*, 1989), any non-functional light chain sequence cloned out of this population is likely to be the cause of the $sIgM^-$ phenotype. Seven of the 17 light chain gene sequences from the $sIgM^-$ subclones encoded frameshifts (Figure 5). Six contained single base pair additions or deletions for which no potential pseudogene donors are known. In one case [CL20 (−)], a deletion of 23 base pairs including the previous 15 base pairs insertion of pseudogene V8 was responsible for the frameshift.

To characterize the phenomenon of sIgM re-expression, $sIgM^+$ cells of CL18 and CL20 subclones were enriched and light chain genes of presumed revertants were determined. As expected for repair by gene conversion events, pseudogene sequences had replaced the out of frame sequences (Figure 6). Most sequences of the CL18 $sIgM^+$ cells contained pseudogene V8-like sequences (eight out of 11 sequences) and it is possible that pre-existing homology favoured conversion events by this donor. Consistent with this view, no preferential usage of the V8 pseudogene was observed for repair of the defective CL20(−) sequence in which all traces of the previous V8 conversion event had been deleted. In two sequences [CL20(+).1 and CL20(+).4] the frameshift of the CL20(−) sequence did not seem to be replaced by pseudogene sequences, but was corrected by deletion of an additional base pair. This may reflect difficulties to convert a sequence that is in this region 8 bp shorter than even the shortest pseudogene. No pseudogene donor is known for the direct repeat of base pairs 113−129 found in another CL20(+) sequence [CL20(+).6].

Discussion

This sequence analysis confirms that clonally derived ALV-induced B cell lymphomas continue to diversify their light

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                     120                                           130         140         150         160         170
Germline     TCC GGG GAT AGC AGC TAC                     TAT GGC TGG TAC CAG CAG AAG GCA CCT GGC AGT GCC CCT GTC
CL16.1       --- --- -G- G-- --- --T GCT GGA AGT TAC TAT --- --- --- --- --- --- --- T-T --- --- --- --- --- ---

CL 2(-)      --- --- -G- G-- -A- --T --- --- --- --- --- --- --- --* C-- --- --- --- T-T --- --- --- --- --- ---
CL 4(-)      --- --- -G- G-- -A- --T --- --- --- --- --- --- --- --- -*- --- --- --- T-T --- --- --- --- --- ---
CL12(-)      --- --- -G- G-- --- ---↓G--- --- --- --- --- --- --- --- --- --- --- T-T --- --- --- --- --- ---
CL14(-)      --- --- -G- G-- -A- --T --- --- --- --- --- --- --T *AG C-- --- --- --- T-T --- --- --- --- --- ---
CL17(-)      --- --- -G- G-- -A- --T --- --- --- --- --- --- --- --- --- --- --- --- T-T --- --- --- -*- --- ---
CL18(-)      --- --- -G- G-- --- ---↓G--- --- --- --- --- --- --- --- --- --- --- T-T --- --- --- --- --- ---
CL20(-)      --- --- -G- G*                              --- --- --- --- --- --- --- T-T --- --- --- --- --- ---
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Fig. 5. Light chain sequences obtained from predominantly sIgM$^-$ subclones. The CL16.1 sequence is shown as a prototype sequence of cells from the sIgM$^+$ bulk population. The starts of frameshifts are indicated by stars (deletions) or arrows (insertions). Each frameshift was confirmed by sequence analysis of a second independent M13 clone.

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                     120                                           130         140         150         160         170    Pseudogene
Germline     TCC GGG GAT AGC AGC TAC                     TAT GGC TGG TAC CAG CAG AAG GCA CCT GGC AGT GCC CCT GTC     donor
CL16.1       --- --- -G- G-- --- --T GCT GGA AGT TAC TAT --- --- --- --- --- --- --- T-T --- --- --- --- --- ---
CL18(-)      --- --- -G- G-- --- ---↓G--- --- --- --- --- --- --- --- --- --- --- T-T --- --- --- --- --- ---

CL18(+).1    --- --- -G- G-- --- --T --- --- --- --- --- --- --- --- --- --- --- --- T-T --- --- --- --- --- ---     V8
CL18(+).2    --- --- -G- G-T --- --T --- --- --- --- --- --- --- --- --- --- --- --- T-T --- --- --- --- --- ---     V8 ?
CL18(+).3    --- --- AG- --T G-- AG-                     --- --- --- --T --- --- --- T-- --- --- --- --- --- ---     V7
CL18(+).4    --- --- AG- GA- --- AG- TAT GGT             --C A-- --- C-- --- --- --- T-T --- --- --- --- --- ---     V4
CL18(+).5    --- --- -G- G-T --- AGC AGC TAC             --- --- --- --- --- --- --- T-T --- --- --- --- --- ---     V5

                     120                                           130         140         150         160         170
Germline     TCC GGG GAT AGC AGC TAC                     TAT GGC TGG TAC CAG CAG AAG GCA CCT GGC AGT GCC CCT GTC
CL16.1       --- --- -G- G-- --- --T GCT GGA AGT TAC TAT --- --- --- --- --- --- --- T-T --- --- --- --- --- ---
CL20(-)      --- --- -G- G*                              --- --- --- --- --- --- --- T-T --- --- --- --- --- ---

CL20(+).1    --- --- -GC                                 --- --- --- --- --- --- --- T-T --- --- --- --- --- ---     ?
CL20(+).2    --- --- -G- G-T G-- AG-                     --- --- --- --T --- --- --- T-- --- --- --- --- --- ---     V10?
CL20(+).3    --- --- -G- --- --T GG- TGT GGT             --- --- --- --T --- --- --- T-T --- --- --- --- --- ---     V12
CL20(+).4    --- --- -C-                                 --- --- --- --- --- --- --- T-T --- --- --- --- --- ---     ?
CL20(+).5    --- --- AG- --T G-- AG-                     --- --- --- --T --- --- --- T-- --- --- --- --- --- ---     V7
CL20(+).6    --- --- -G- -TG GCT GT- CGG GGG             --- --- --- --- --- --- --- T-T --- --- --- --- --- ---     ?
CL20(+).7    --- --- AG- GA- --- AG- TAT GGT             -GC A-- --- C-- --- --- --- T-T --- --- --- --- --- ---     V4 ?
CL20(+).8    --- --- -GC --- TAT AG-                     --- --- --- --- --- --- --- T-T --- --- --- --- --- ---     V14
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Fig. 6. Light chain gene sequences obtained from sIgM$^+$ cells of the CL18 and Cl20 subclones. Some sequences were found more than once. For example the CL18(+).1 sequence was found 7 times between the 11 sequences determined from IgM$^+$ cells of the CL18 subclone.

chain immunoglobulin genes (Thompson and Neiman, 1987; Thompson *et al.*, 1987). As in bursal B cells, diversification is likely to occur by gene conversion events with pseudogene sequences serving as donors. On-going gene conversion within tumor cells is not dependent upon the bursal environment, since it continued in a thymic metastasis and during the *in vitro* culture of the cell line DT40.

The sequence modifications observed in random DT40 subclones corresponded mainly to substitutions of single or a few base pairs, while longer converted segments could be seen in bursal B cells after a similar number of divisions. Thus the estimated rate of gene conversion events in DT40 subclones may not be strictly comparable to the rate found in bursal B cells. Since longer conversion tracts were seen among the sequences of the selected DT40 sIgM$^+$ revertants (see below), they do occur, but presumably at a lower frequency. No light chain gene heterogeneity was found in several other ALV-induced tumor cell lines. This lack of detectable conversion activity may be due to cell differentiation or loss of chromosomes, and it is possibly linked to the way these cell lines were established (Baba *et al.*, 1985; Ewert and Weber, 1987).

Assuming the sIgM expression is not required for the survival of transformed cells, we searched for non-functional sequences within sIgM$^-$ cells of the DT40 cell line. After enrichment and subcloning of a small subpopulation ($\sim 1\%$) of sIgM$^-$ cells, we could identify frameshifts within the light chain genes of several of these sIgM$^-$ subclones. In most cases, the frameshifts are due to single bases pair additions or deletions that could not easily be correlated with sequences found in the pseudogene pool. In one case [CL20($-$)], a large deletion was responsible for the loss of the reading frame. In the light chain genes of subclone 16 (CL16.1$-$CL16.4) a deletion was found in the middle of the second framework region at the site of a direct repeat (GACAACGACAA), but it involved precisely two codons apparently not interfering with light chain expression.

A small number of cells within most of the sIgM$^-$ subclones spontaneously re-expressed sIgM. For the two subclones studied most of these revertants could be explained by repair of the light chain frameshifts through gene conversion events by donors in the pseudogene pool. In some cases, repair involved single base pair modifications which were not assignable to a gene conversion event.

It is surprising that none of the encountered light chain gene defects can be easily explained by attempted conversion events, but mainly involved untemplated single base pair deletions or additions. Untemplated base substitutions have been observed among diversified bursal light chain sequences at the putative borders of the converted segments (Reynaud *et al.*, 1987). These single base pair modifications which may occur at low frequency as part of the conversion mechanism would be expected to be more error prone than the templated events. In some cases poor alignment between donor and acceptor sequences might induce such untemplated events. For instance, the five codon insertion from pseudogene V8 present in the CDR1 of most sequences from DT40 as well as the large deletion found in the CL20(−) sequence might impair the alignment of pseudogenes during further conversion attempts.

The low percentage of $sIgM^-$ cells within the DT40 bulk population suggest that the conversion mechanism operates at a low error rate in chicken bursal cells. The proposed high death rate of these cells *in situ* (Lassila, 1989) is probably caused by B cell repertoire selection, rather than by errors in the gene conversion process. Whether the repair of frameshifts during the conversion process plays a role in the rescue of bursal B cells with non-functional light chain genes remains open. However, this repair mechanism may dictate the unique immunoglobulin gene configuration found in chicken B cells: one productively rearranged allele and the other allele remaining in germline configuration (Reynaud *et al.*, 1985; McCormack *et al.*, 1989). A mammalian type of immunoglobulin gene configuration would result in frequent 'allelic inclusion', the conversion mechanism being able to put abortive rearrangements back into frame.

The demonstration that light chain gene conversion continues during *in vitro* culture of the cell line DT40 opens new possibilities for investigation. Transfection of DT40 with modified versions of the light chain locus may help to clarify the sequence requirements of this recombination phenomenon.

Materials and methods

ALV lymphoma induction

The chickens used in this study were from a cross between two highly inbred lines, $15I_5$ and 7_1. Fertile eggs were obtained from the Regional Poultry Research Laboratory (RPRL), East Lansing, MI. The eggs were hatched and the chicks reared in The Wistar Institute Animal Facility under conditions which meet the standards of the US Department of Agriculture. Chickens were infected at hatching with 0.1 ml of culture supernatant containing 10^4 infectious units of RAV-1, a subgroup-A ALB. This virus was obtained from L.B.Crittenden (RPRL), and grown to high titer in fibroblasts from line 0 embryos (Astrin *et al.*, 1979).

Cell culture

The ALV-induced LSCC-DT40 cell line (DT40) (Baba *et al.*, 1985) was used following 10th passages *in vitro*. This cell line as well as the LCSS-RP9 cell line (Okazaki *et al.*, 1980) and the newly established ALV-induced cell lines were cultured in modified Dulbecco's medium containing 10% fetal calf serum, 1% chicken serum, 2 mM L-glutamine, 10^{-5} M β-mercaptoethanol and penicillin/streptomycin. Subcloning was by limiting dilution.

Sequence analysis

Genomic DNA from tumors and cell lines was isolated using conventional techniques. The PCRs were performed using the gene amplification kit of the Cetus Corporation (Perkin Elmer Cetus) following the manufacturer's instructions except that reaction volumes were reduced to 30 μl. Since the first 30 cycles resulted in unsatisfactory amplification, a second round of amplification was performed using the upstream primer and a second nested downstream primer. The upstream primer was derived from the intron sequence GGGCAGGGCTGTGCGTGC between the light chain leader exon and the V gene segment (Reynaud *et al.*, 1987), whereas the first downsteam primer and the nested downstream primer was complementary to unpublished sequences (C.-A.Reynaud) downstream of the J gene segment (complementary sequences of primers: AGCCTGCCGCCAAGTCC and CGACAAAATGTCACAATTTCACG). Primers encoded additional *Eco*RI and *Xba*I restriction sites at their 5′ ends and these sites were used for subsequent cloning of the amplified sequence into M13 phage. Single stranded sequencing of M13 clones was performed using Sequenase (United States Biochemical) according to manufacturer's instructions.

Staining for sIgM

Staining for the presence of sIgM was done with the mouse anti-chicken IgM mAb M-1 (Chen *et al.*, (1982) (kindly provided by C.-L.Chen, University of Alabama). Approximately 10^5 cells were incubated for 30 min on ice in 50 μl of a 1/500 dilution of ascites, containing the M-1 mAb in incubation buffer (phosphate-buffered saline, 0.3% bovine serum albumin, 0.02% sodium azide). After two washes, cells were incubated for 30 min on ice in a 1/100 dilution of FITC labeled $(Fab')_2$ fragments of sheep anti-mouse immunoglobulin (Silenus, Hawthorn, Australia). After two further washes cells were resuspended in 0.5 ml buffer and analyzed by a FACSCAN instrument (Becton-Dickinson, Mountain View, CA). Negative controls were performed by incubating cells with buffer alone followed by incubation with FITC conjugate.

For enrichment of $sIgM^-$ cells, 5×10^7 DT40 cells were incubated with M-1 mAb and subsequently treated with rabbit complement (Low tox rabbit complement, Cedarlane, Ontario) according to manufacturer's instructions. Complement treatment after mAb incubation resulted in 90% cell death, whereas complement incubation alone killed 50% of the cells. Surviving cells were expanded in culture to 5×10^7 cells and the complement-mediated cell killing was repeated. Cells surviving the second treatment were stained with M-1 mAb and negative cells were sorted by a FACS 440 instrument. This sorted $sIgM^-$ population was then subcloned by limiting dilution.

Revertant $sIgM^+$ cells from two predominantly $sIgM^-$ subclones (Cl.18 and C1.20) were enriched by the use of sheep anti-mouse IgG coated Dynabeads (Dynal, Norway) after staining the cells with M-1 mAb.

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