Retroviral Transformation In Vitro of Chicken T Cells Expressing Either α/β or γ/δ T Cell Receptors by Reticuloendotheliosis Virus Strain T

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Summary

Exposure of normal juvenile chicken bone marrow cells to the replication defective avian reticuloendotheliosis virus strain T (REV-T) (chicken syncitial virus [CSV]) in vitro resulted in the generation of transformed cell lines containing T cells. The transformed T cells derived from bone marrow included cells expressing either α/β or γ/δ T cell receptors (TCRs) in proportions roughly equivalent to the proportions of TCR-α/β and TCR-γ/δ T cells found in the normal bone marrow in vivo. Essentially all TCR-α/β-expressing transformed bone marrow–derived T cells expressed CD8, whereas few, if any, expressed CD4. In contrast, among TCR-γ/δ T cells, both $CD8^+$ and $CD8^-$ cells were derived, all of which were $CD4^-$. Exposure of ex vivo spleen cells to REV-T(CSV) yielded transformed polyclonal cell lines containing >99% B cells. However, REV-T(CSV) infection of mitogen-activated spleen cells in vitro resulted in transformed populations containing predominantly T cells. This may be explained at least in part by in vitro activation resulting in dramatically increased levels of T cell REV-T(CSV) receptor expression. In contrast to REV-T(CSV)–transformed lines derived from normal bone marrow, transformed lines derived from activated spleen cells contained substantial numbers of $CD4^+$ cells, all of which expressed TCR-α/β. While transformed T cells derived from bone marrow were stable for extended periods of in vitro culture and were cloned from single cells, transformed T cells from activated spleen were not stable and could not be cloned. We have therefore dissociated the initial transformation of T cells with REV-T(CSV) from the requirements for long-term growth. These results provide the first demonstration of efficient in vitro transformation of chicken T lineage cells by REV-T(CSV). Since productive infection with REV-T(CSV) is not sufficient to promote long-term growth of transformed cells, these results further suggest that immortalization depends not only upon expression of the v-*rel* oncogene but also on intracellular factor(s) whose expression varies according to the state of T cell physiology and/or activation.

Reticuloendotheliosis virus strain T (REV-T)[1] is a replication-defective avian retrovirus, first characterized as inducing acute reticuloendothelial neoplasia in vivo (1, 2). REV-T contains the transforming v-*rel* oncogene inserted within its *env* gene (3, 4), although the molecular basis for cell transformation by v-*rel* is currently unclear. REV-T requires a helper virus for replication, the most widely used of which has been the REV-A retrovirus (5). However, REV-A itself is suppressive for lymphoid cells in vivo (6, 7), and infection in vivo or in vitro with REV-T(REV-A) has led to transformation of target cells frequently defined as being immature cells of hematopoetic origin (8–10). Subsequently, chicken syncitial virus (CSV) has been used as a helper virus for REV-T, and the resulting REV-T(CSV) has proved to be highly efficient in the induction of lymphocyte transformation in vivo (11, 12) or in vitro (13, 14). Exposure of chickens in vivo leads to the rapid induction of polyclonal B cell tumors that can readily be adapted to in vitro growth (11, 12). Similarly, exposure of lymphoid cells in vitro to REV-T(CSV) also leads to polyclonal B cell transformation (13). While some reports have suggested that REV-T(REV-A)–transformed B lineage cells may undergo loss or changes within the Ig loci during in vitro culture (15–17), REV-T(CSV)–transformed B lineage clones induced in vivo or in vitro have stable Ig loci during extended periods of in vitro growth (11, 13). Recently, exposure of B cell–deficient chickens to REV-T(REV-A) in vivo led to the generation of T cell tumors in vivo (18), suggesting that REV-T-based viruses might provide a means of transforming at least some subsets of chicken T cells.

Avian species have provided valuable models for studies

[1] *Abbreviations used in this paper:* CSV, chicken syncitial virus; REV, reticuloendotheliosis virus strain.

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of lymphoid cell development from immature precursors largely as a consequence of the accessibility of the embryo to surgical manipulation (e.g., reference 19). The degree of conservation of T cell developmental biology (20, 21) and cell surface antigen expression (22–29) between avian and mammalian species is extremely high with the result that the analysis of avian T cell development has general relevance to mammalian as well as avian species. One drawback of avian models of T cell development and activation has been a relative paucity of defined transformed cells, compared with mammalian species, as sources of material for the analysis of the biochemistry of cell surface molecules and their interactions.

In this report we demonstrate that transformed T cells can be derived from exposure of normal ex vivo or activated normal chicken T cells to REV-T(CSV) in vitro. Such T cells express either TCR-α/β or -γ/δ, the latter representing the first stable isolation of transformed chicken γ/δ T cells. Whereas REV-T(CSV) transformation of ex vivo spleen cells generated cell lines containing exclusively B cells, exposure of mitogen-activated spleen cells to REV-T(CSV) resulted in transformed lines containing predominantly T cells, and including both $CD4^+$ and $CD8^+$ cells. While transformed T cell lines derived from mitogen-activated spleen cells were unstable, lines derived from normal bone marrow could be cloned with high efficiency, and T cell clones of either α/β or γ/δ lineage were generated. Consequently, while the activity of the v-*rel* oncogene is functionally expressed in T cells, parameters other than v-*rel* expression limit the immortalization of chicken T cells by REV-T.

Materials and Methods

Cells. Bursa, spleen, bone marrow, and PBL were prepared from 6–8-wk-old SC chickens (Hyline International, Dallas Center, IA) as described elsewhere (29). Spleen T cells were prepared by incubation of normal spleen cells with the anti-Bu-1 antibodies 21-1A4 and Fu5.11G2 (30) for 15 min followed after washing by incubation at 37°C for 30 min with rabbit anti-mouse Ig and guinea pig complement (Cedarlane Laboratories, Hornby, Ontario, Canada), each preabsorbed on chicken lymphoid cells.

Tissue Culture. All tissue culture was performed in IMDM supplemented as described elsewhere (13). Supernatant from the S_2A_3 cell line was filtered, aliquotted, stored at -70°C, and thawed immediately before use as a source of REV-T(CSV) (13). Spleen cells were activated by culture for 3–4 d at 5×10^6 cells/ml in the presence of 3 μg/ml Con A (Sigma Chemical Co., St. Louis, MO). Con A was subsequently removed by washing cells in 0.05 M methyl α-D-mannopyrannoside (Sigma Chemical Co.) before further use.

Cells were transformed by culture at $2–5 \times 10^6$/ml in the presence of 75% S_2A_3 supernatant. When cell growth was evident, cells were passaged in IMDM without further addition of virus-containing supernatants. Cell lines were cloned by growth from microscopically observed single cells in 10-μl Terrasaki cultures established in the absence of any exogenous filler cells, as described elsewhere (13).

The frequency of bone marrow cells transformed by REV-T(CSV) was established by colony formation in 15 ml 0.3% Bacto-Agar (Difco Laboratories, Detroit, MI) in IMDM in 8.5-cm-diameter petri dishes containing between 2×10^5 and 1×10^7 cells in the presence of 75% S_2A_3 supernatant. Transformation efficiency was measured by quantitation of colonies visible to the naked eye after 8–11 d of culture. The frequency of splenic T cells transformed by REV-T(CSV) was established by limiting dilution analysis in 10-μl Terrasaki cultures containing titrated numbers of splenic T cells and 75% S_2A_3 supernatant. Transformation was defined by growth in 10-μl cultures requiring passage to 100-μl cultures within 7 d.

Antibodies and Flow Cytometric Analysis. The mAbs used in these studies were as follows: 6E1, anti-chicken Ig light chain (31); EP96, anti-chicken CD4 (29); EP72, anti-chicken CD8 (29); CT3, anti-chicken CD3 (22); TCR1, anti-chicken TCR-γ/δ (23); TCR2, anti-chicken TCR-α/β (24), defining those T cells using the Vβ1 gene (32); and TCR3, anti-chicken TCR-α/β (25), defining those T cells using the Vβ2 gene (32). Binding of primary antibodies was detected using either FITC-conjugated monoclonal rat anti-mouse κ (187.1) (33) as described elsewhere (29) or using FITC- or R-PE-conjugated goat anti-mouse Ig isotype antibodies (Southern Biotechnology Associates, Birmingham, AL). Viable cells were analyzed on a FACScan® (Becton Dickinson Canada Inc., Mississauga, Ontario, Canada) by gating on forward and side scatter.

Southern Blot Analysis. Cellular DNA was prepared from $\sim 4 \times 10^6$ cells/track and digested with a 10-fold excess of restriction enzymes according to manufacturer's instructions (Pharmacia, Baie D'Urfé, Quebec, Canada). Digested DNA was run on 0.8% agarose gels before depurination, denaturation, blotting onto nylon membranes, and UV crosslinking. Blots were probed with a nick-translated probe comprising a 967-bp EcoRI fragment of the PKW101 plasmid, which hybridizes to v-*rel* and c-*rel* sequences (34), incorporating α-[^{32}P]dCTP (ICN Biomedicals Canada, Ltd., Toronto, Ontario, Canada) to a specific activity of between 6×10^8 and 2×10^9 cpm/μg DNA as described elsewhere (13).

Results

REV-T(CSV) Transforms Chicken T Cells Expressing Either TCR-α/β or -γ/δ. Exposure of chickens in vivo to the REV-T(CSV) retrovirus leads to the rapid induction of polyclonal B cell lymphomas that can readily be adapted to in vitro growth (11, 12). More recently, however, exposure of cyclophosphamide-treated (B cell–deficient) chickens to REV-T(REV-A) resulted in the induction of T cell tumors in vivo (18). We have shown that transformed cells expressing surface Ig can be isolated and cloned from embryo bone marrow after exposure to REV-T(CSV) in vitro (13). However, infection of cells from embryo bone marrow with REV-T(CSV) induced transformation of not only sIg^+ cells but a substantial population of sIg^- cells (13) whose lineage is currently unclear but nonetheless demonstrated that sIg expression is not a prerequisite for REV-T(CSV) transformation in vitro.

Exposure of unfractionated lymphocytes from spleen, bursa, or peripheral blood of 3–8-wk-old normal chickens to REV-T(CSV) in vitro resulted in the generation of transformed cells, essentially all of which expressed sIg (Fig. 1). In contrast, exposure of bone marrow cells from 6-wk-old chickens to REV-T(CSV) resulted in the generation of cell lines that routinely contained considerably <100% sIg^+ cells. Typically, 2 wk after initiation, one such culture contained $\sim$37% of sIg^+ cells (Fig. 1 *d*) and was clearly polyclonal as judged by the multiple foci of cell growth in the primary cultures.

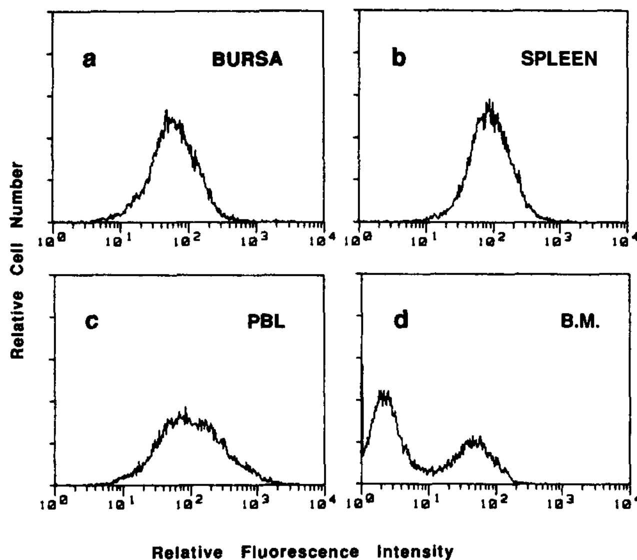

Figure 1. REV-T(CSV) transformation of sIg⁻ cells from chicken bone marrow. Spleen, bursa, PBL, and bone marrow cell suspensions from normal chickens were transformed with REV-T(CSV) in the presence of PMA. Transformed cells from: (*a*) bursa, 14 d after transformation; (*b*) spleen 12 d after transformation; (*c*) PBL, 14 d after transformation; and (*d*) bone marrow, 14 d after transformation were stained with 6E1 (anti-chicken Ig light chain) followed by FITC-conjugated 187.1. Staining profiles from 10,000 viable cells are shown.

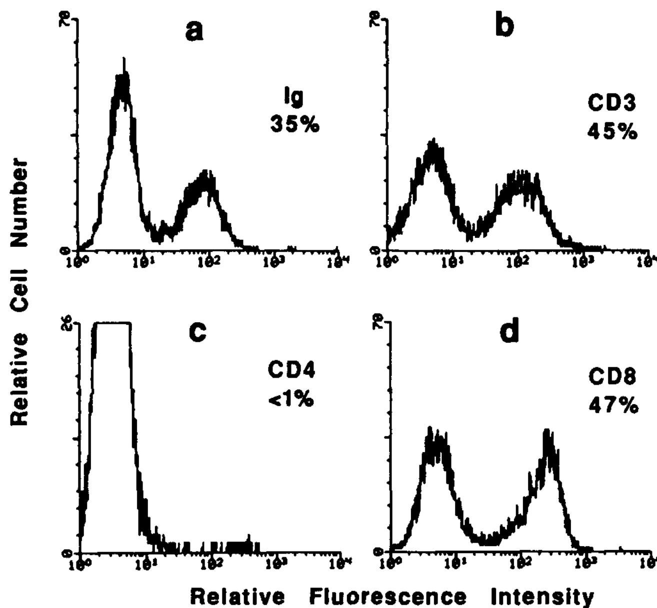

Figure 2. Presence of T cells among REV-T(CSV)-transformed chicken bone marrow cells. A REV-T(CSV)-transformed chicken bone marrow cell line was stained 6 wk after transformation with: (*a*) 6E1 (anti-chicken Ig light chain) followed by FITC-conjugated 187.1; (*b*) CT3 (anti-chicken CD3) followed by PE-conjugated anti-mouse IgG1; (*c*) EP96 (anti-chicken CD4) followed by FITC-conjugated anti-mouse IgM; or (*d*) EP72 (anti-chicken CD8) followed by FITC-conjugated anti-mouse IgG2b. Staining profiles from 10,000 viable cells are shown.

Each of the REV-T(CSV)-transformed cell lines described in Fig. 1, including the line from bone marrow, contained exclusively cells expressing high levels of MHC class II (detected with the 21-1A6 mAb [35]) and undetectable levels of the Bu-1 B cell surface antigen (detected with the Fu5.11G2 mAb [30]) (data not shown). This is consistent with the observed phenotype of cells transformed with REV-T(CSV) in vivo (11) or in vitro (13).

Another similarly transformed cell line derived from juvenile bone marrow contained ~35% of sIg⁺ cells 6 wk after transformation and was further analyzed for the expression of T cell surface antigens (Fig. 2). Approximately 45% of cells within this line expressed the CD3 complex as determined by the CT3 mAb (Fig. 2 *b*). Fewer than 1% of cells expressed the CD4 accessory molecule, whereas 47% of cells expressed CD8 (Fig. 2, *c* and *d*).

To estimate the frequency of cells transformed by REV-T(CSV), titrated numbers of bone marrow cells were cultured in soft agar in the presence of virus. The formation of macroscopic colonies was dependent on the presence of REV-T(CSV), and the frequency of bone marrow cells transformed by the virus was ~1 in 500–1,000 (Table 1). Since the majority of cells transformed by REV-T(CSV) were either CD3⁺ or sIg⁺, and these cells represent ~5% of the ex vivo bone marrow cell suspensions, as judged by flow cytometry, the frequency of bone marrow lymphoid cells transformed can be estimated at ~1 in 20–40.

Chicken T cells, as is the case in mammalian species, can be divided into those expressing TCR-α/β heterodimers and those expressing TCR-γ/δ heterodimers (20). 8 wk after transformation, the bone marrow–derived cell line described in Fig. 2 contained 15% cells expressing TCR-γ/δ as defined by the TCR1 (23) mAb (Fig. 3 a, *y-axis*). The chicken TCR

Table 1. *Frequency of Bone Marrow Cells Transformed by REV-T(CSV)*

		Colonies*	
Cell number	REV-T(CSV)‡	Per plate	Per 10^2 lymphocytes§
2×10^6	+	3,300	3.2
2×10^6	+	2,600	2.6
2×10^6	–	<10	–
6×10^5	+	1,600	5.4
6×10^5	–	<10	–
2×10^5	+	650	6.4
2×10^5	–	<10	–

* Macroscopic colonies were counted after 8–11 d of culture.
‡ Bone marrow cells were cultured in the presence of 75% S_2A_3 supernatant in 15-ml soft agar cultures.
§ Colonies per 10^2 lymphocytes were calculated from the number of colonies per plate by accounting for the percentage of lymphocytes in the starting bone marrow cell populations (typically 3–5%).

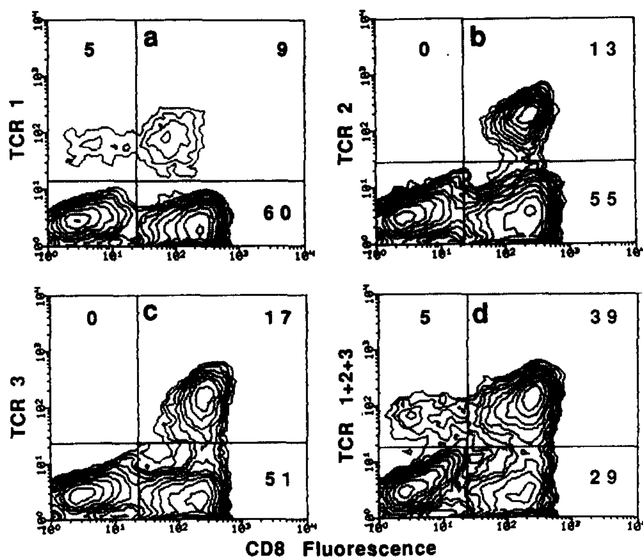

Figure 3. Presence of α/β and γ/δ T cells in REV-T(CSV)–transformed bone marrow cells. The cell line described in Fig. 2 was stained after 8 wk of culture with: (*a*) TCR1 (anti-TCR-γ/δ) and EP72 (anti-CD8); (*b*) TCR2 (anti-TCR Vβ1) and EP72; (*c*) TCR3 (anti-TCR Vβ2) and EP72; or (*d*) a mixture of TCR1, TCR2, TCR3, and EP72. TCR1, TCR2, and TCR3 were each detected with PE-conjugated anti-mouse IgG1, and EP72 was detected with FITC-conjugated anti-mouse IgG2b. Contour plots from 20,000 viable cells are shown. The numbers in each panel represent the percentage of cells falling within the respective quadrant.

β chain locus contains two Vβ genes, Vβ1 and Vβ2 (36). All T cells expressing the Vβ1 gene as part of a TCR-α/β complex are recognized by the TCR2 mAb, and all T cells expressing Vβ2 are recognized by the TCR3 mAb (32). Flow cytometric staining of the REV-T(CSV)–transformed bone marrow cell line revealed 13% V$\beta 2^+$ cells (Fig. 3 *b*, *y-axis*) and 17% V$\beta 3^+$ cells (Fig. 3 *c*, *y-axis*). The frequency of $CD3^+$ cells within this line at this time was 45%, consistent with the finding that the TCR1, TCR2, and TCR3 antibodies stained mutually exclusive populations of cells within this line, which together accounted for all the surface $CD3^+$ cells (data not shown), as they do in normal ex vivo chicken T cells (25). The relative proportions of α/β- and γ/δ-expressing T cells within the line were approximately equivalent to the relative proportions of these cells in the normal chicken. It is clear therefore that REV-T(CSV) can productively infect in vitro and transform chicken T cells of both α/β and γ/δ lineages.

All TCR-α/β^+ ($TCR2^+$ and $TCR3^+$) T cells within the bone marrow–derived line expressed high levels of the CD8 accessory molecule (Fig. 3, *b* and *c*). In contrast, TCR-γ/δ ($TCR1^+$) T cells were heterogeneous for the expression of CD8 (Fig. 3 *a*). Specifically, about one-third of TCR-γ/δ cells were $CD8^-$, and two-thirds were $CD8^+$. This is consistent with the distribution of CD8 on peripheral γ/δ T cells in the normal chicken (22), and demonstrates that despite the expression of CD8 on essentially all transformed bone marrow α/β T cells, the expression of CD8 is not required for REV-T(CSV)–mediated T cell transformation.

Transformation of $CD3^-/CD8^+$ Cells. Since the proportion of $CD8^+$ cells (68%) within this transformed bone marrow line at 8 wk after transformation exceeded the number of cells staining with a combination of the three TCR mAbs (a total of 45%; Fig. 3, *a–c*) or with the CT3 anti-CD3 antibody (data not shown), and further, that some γ/δ T cells were $CD8^-$, it seemed probable that the line contained $CD8^+$ cells that did not express surface TCR. This was demonstrated directly in Fig. 3 *d*, where 29% of cells within the line were $TCR^-,CD8^+$. It is possible that these cells represent the transformed counterparts of the TCR0 cells found in the normal chicken that have been characterized as surface $TCR^-,CD3^-$ but contain CD3 component(s) cytoplasmically, as detected by staining with the CT3 antibody on fixed cells (37). At least some TCR0 cells express the CD8 molecule (38), consistent with the surface $CD3^-$, $CD8^+$ phenotype of some cells within the bone marrow line.

Transformation of $CD4^+$ T Cells from Activated Spleen Cell Populations. The transformation of T cells from bone marrow was surprizing since juvenile chicken bone marrow typically contains <5% T cells compared with 40–60% T cells in the spleen. We considered the possibility therefore that the relative susceptibility of bone marrow T cells to REV-T(CSV)–mediated transformation was a reflection of differences in T cell physiology compared with splenic T cells. Spleen cells were therefore stimulated for 3 d in vitro with Con A, after which time such populations routinely contained >95% $CD3^+$ cells, ~20% $CD4^+$ cells, ~75% $CD8^+$ cells, and <3% sIg^+ B cells. Con A–activated spleen cells were then cultured in the presence of REV-T(CSV)–containing supernatants without any further stimulus. Continued and extensive cell growth occurred in cultures containing virus, whereas cultures not containing virus had no viable cells after 48–72 h. As before, transformed cell growth was clearly polyclonal and rapid. Staining this population 12 d after transformation revealed ~10% TCR-γ/δ cells, all of which were $CD4^-$ and about half of which were $CD8^+$ (Fig. 4), similar to the phenotypes of TCR-γ/δ^+ cells transformed from juvenile chicken bone marrow.

55% of cells within the line expressed TCR-α/β using the Vβ1 gene ($TCR2^+$). Of these, about one-quarter (24%) expressed CD4, and three-quarters (76%) expressed CD8. Similarly, of the 30% of cells expressing TCR-α/β using the Vβ2 gene ($TCR3^+$), 25% expressed CD4, and 75% expressed CD8 (Fig. 4). Double staining revealed that CD4 and CD8 in this line were expressed on mutually exclusive populations of cells (data not shown). Consequently, it is clear that REV-T(CSV) can transform $CD4^+$ as well as $CD8^+$ T cells, and that the $CD4^+$ population of cells transformed is restricted to those expressing TCR-α/β.

The frequency of Con A–activated splenic T cells transformed by REV-T(CSV) was estimated by limiting dilution in 10-μl Terrasaki cultures containing 75% S_2A_3 supernatant. Regression analysis of the limiting dilution (Fig. 5) demonstrated that ~1 in 420 activated splenic T cells was transformed by REV-T(CSV). As before, growth was completely dependent on the presence of virus.

Activation-induced Receptors for REV-T(CSV) on T Cells. It

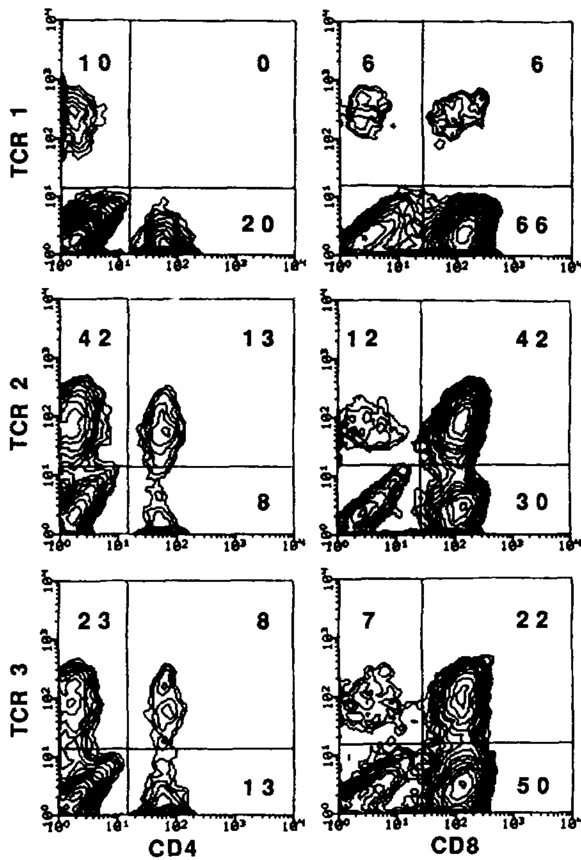

Figure 4. REV-T(CSV) transformation of $CD4^+$ and $CD8^+$ cells from activated spleen Transformed cells derived by exposure of activated spleen cells to REV-T(CSV) were cultured for 14 d and stained for the expression of TCR, CD4, and CD8 Cells were stained with either TCR1 (anti-TCR-γ/δ), TCR2 (anti-Vβ1 TCR), or TCR3 (anti-Vβ2 TCR), each being detected using PE-conjugated anti-γ1, in conjunction with either EP96 (anti-CD4) or EP72 (anti-CD8), which were detected with FITC-conjugated anti-μ or FITC-conjugated anti-γ2b, respectively Contour plots from 20,000 viable cells are displayed, and the numbers in each panel represent the percentage of cells falling within each quadrant

seemed possible that the failure to efficiently transform ex vivo splenic T cells with REV-T(CSV) might be, at least in part, a consequence of a lack of T cell surface expression of a receptor for the virus. Consistent with this hypothesis, purified splenic T cells (>95% $CD3^+$) were unable to absorb the transforming activity from REV-T(CSV)-containing supernatants (Fig. 6), as assayed by the ability of absorbed supernatants to subsequently transform ex vivo bursal cells. In contrast, Con A-activated spleen cells, after washing in 0.05 M methyl α-D-mannopyrannoside to remove cell-bound Con A, absorbed transforming activity more efficiently than did ex vivo bursal cells, an established target of REV-T(CSV) transformation. Consequently, activation of splenic T cells induced expression of functional receptors for the REV-T(CSV) retrovirus.

Stability and Cloning of REV-T(CSV)-transformed T Cells. The transformed lines derived from bone marrow were relatively stable over extended periods of time in vitro. Changes

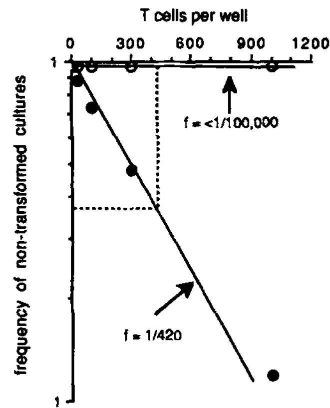

Figure 5. Frequency of activated spleen cells transformed by REV-T(CSV) Titrated numbers of mitogen-activated T cells were cultured in 10-μl Terrasaki in the presence (●) or absence (○) of REV-T(CSV) 10-μl cultures (144/point) were scored as positive when cell growth completely covered the bottom of the well (f) Frequency of cells transformed with REV-T(CSV), corresponding to the input cell number at which 37% of cultures contained no cell growth

in the relative proportion of phenotypically distinct cells were consistent with small differences in growth rates among the different cell populations within the lines. In contrast, transformed lines derived from Con A-activated spleen were not stable. After ~2-3 wk of growth, the frequencies of $CD3^+$, $CD4^+$, and $CD8^+$ cells declined from those seen in Fig. 4 such that by 4-6 wk after transformation the majority of cells within the line were negative for these T cell markers.

To determine whether the T cells transformed from different sources had differing potentials for long-term growth, we established limiting dilution cultures of REV-T(CSV)-trans-

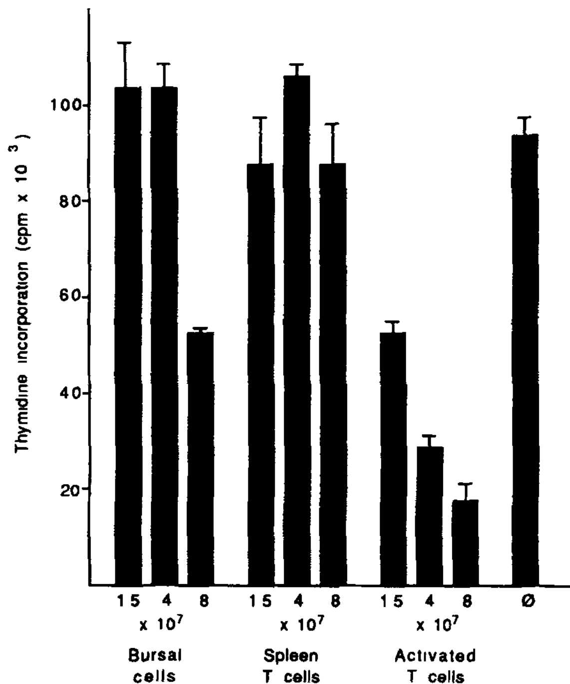

Figure 6. Expression of receptors for REV-T(CSV) on activated T cells 1-ml aliquots of S_2A_3 REV-T(CSV)-containing supernatant were incubated with the indicated number of bursal cells, spleen T cells, Con A-activated spleen T cells, or in the absence of cells (Ø) for 30 min at 37°C. Cells were removed by centrifugation, and the resulting supernatant was cultured at 25% with 6 × 10^4 bursal cells in 200-μl cultures in the presence of 20 ng/ml PMA for 48 h During the last 6 h cultures were pulsed with [^{3}H]thymidine (1 μCi/well), harvested, and counted Means and SDs of triplicate cultures are shown

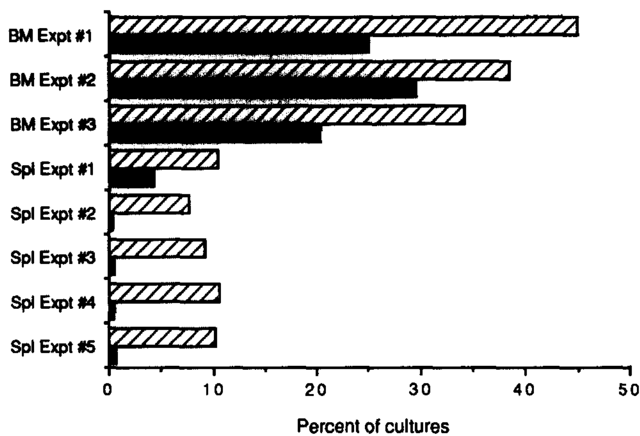

Figure 7. Clonability of REV-T(CSV)-transformed T cells derived from bone marrow or spleen. REV-T(CSV)-transformed cells were plated at a concentration one cell/10 μl in 10-μl cultures in the absence of added filters or virus-containing supernatants. (▨) The percent of cultures with cell growth over the first week of culture; (■) the percent of cultures containing cells that maintained growth subsequent to passage into 200 μl followed by 2-ml cultures.

formed lines derived from normal bone marrow or Con A–activated spleen cells. 10-μl cultures, each containing an average of one cell per culture, were established in the absence of added fillers, and growth was monitored during the first week. Cultures with significant cell growth (100–1,000 cells) as judged microscopically were expanded into 100-μl cultures and subsequently into 2-ml cultures as necessary.

Limiting dilution cultures established from bone marrow showed a high cloning efficiency within the first week of culture (30–45% of wells with growth). The majority of these cultures (>65%) maintained growth subsequent to passage into larger cultures and continued to grow for weeks to months (Fig. 7). Large panels of clones were thereby derived from transformed bone marrow lines, and ~200 clones (those derived from microscopically observed single cells) were examined for cell surface antigen expression. Representative examples of the cloned cell phenotypes are shown in Fig. 8. In general, the relative frequency of clones of a given phenotype corresponded to the proportion of cells with that phenotype in the starting population of cells from which they were cloned. After cloning, the levels of expression of TCR (either α/β or γ/δ) and CD8 were stable for weeks to months thereafter.

In contrast, considerably less short-term growth was observed in limiting dilution cultures established from REV-T(CSV)-transformed Con A–activated spleen cells (Fig. 7). Typically, <15% of the input cells grew over the first week of culture. The proportion of cultures in which continued cell growth was maintained was extremely small (<2%). Among the few cells cloned from these lines, none expressed the T cell markers CD3, CD4, or CD8. This suggests that

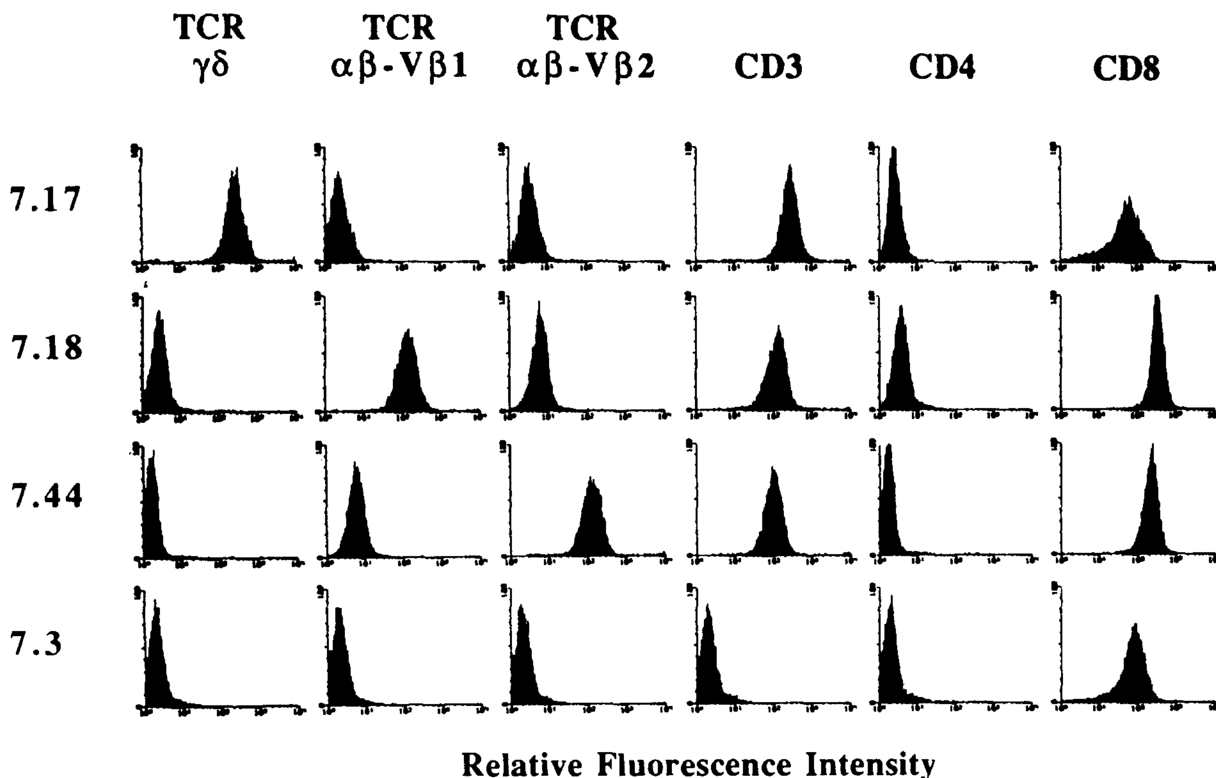

Figure 8. Surface antigen expression among REV-T(CSV)-transformed clones. Clones isolated from REV-T(CSV)-transformed bone marrow as described in the text were stained with TCR1, TCR2, TCR3, CT3 (anti-CD3), EP96 (anti-CD4), or EP72 (anti-CD8), each being detected with the appropriate FITC- or PE-conjugated antiisotype reagent as described. Profiles from 10,000 viable cells are shown.

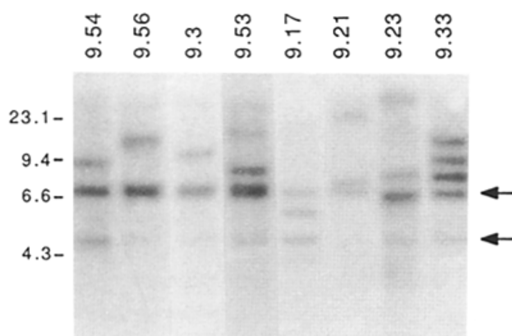

Figure 9. REV-T proviral integration in cloned transformed chicken T cells. DNA from 5 × 10^6 cells from clones of REV-T(CSV)-transformed bone marrow was digested with BclI, subjected to electrophoresis, blotted, and probed with ^{32}P-labeled v-*rel* sequences. The phenotypes of these clones were: lanes *9.54* and *9.56*, TCR-γ/δ^+,CD3$^+$,CD8$^-$; lane *9.3*, TCR-α/β (Vβ1)$^+$,CD3$^+$,CD8$^+$; lane *9.53*, TCR-α/β (Vβ2)$^+$,CD3$^+$, CD8$^+$; lane *9.17*, TCR$^-$,CD3$^-$,CD8$^+$; lanes *9.21*, *9.23*, and *9.33*, TCR$^-$,CD3$^-$,CD8$^-$, c-*rel*-specific bands are indicated with arrows. The numbers on the left refer to molecular size markers (kb).

REV-T(CSV)-transformed T cells derived from Con A-activated spleen cell populations have limited growth potential in the absence of exogenous growth support, and in the polyclonal line were outgrown by transformed non-T cells.

Proviral Integration into the T Cell Genome. REV-T(CSV) is a highly efficient virus for transforming B cells. Since the results demonstrated here represented the first description of efficient T cell transformation by supernatants containing REV-T(CSV), it was important to confirm that the REV-T proviral genome was indeed integrated into the transformed T cell genome. Analysis of the DNA from a panel of clones, derived from REV-T(CSV)-infected bone marrow cells, of a variety of phenotypes (either TCR-α/β^+, TCR-γ/δ^+, or TCR$^-$,CD3$^-$) demonstrated the presence of the integrated REV-T genome as judged by hybridization with the v-*rel* probe (e.g., Fig. 9). Bands at 7 kb and, more weakly, at 4.7 kb (*arrows*) were observed in Bcl-1 digests of all clones (as well as normal cells [13]) and corresponded to the c-*rel* homologue of the v-*rel* oncogene (11). In addition to these bands each clone contained unique bands not present in normal cells, demonstrating the random integration of the REV-T provirus as is seen in B cells transformed with REV-T(CSV) in vivo (11) or in vitro (13). Therefore, T cell transformation can be mediated by productive infection with REV-T(CSV) and consequent REV-T proviral integration and expression of the v-*rel* oncogene.

Discussion

The REV-T(CSV)-transformed clones of chicken T cells derived from bone marrow and expressing either TCR-α/β or -γ/δ heterodimers represent the first demonstration of stable clonal populations of chicken T cells transformed by exposure to REV-T in vitro. Such clones obviously provide an ideal source of material for characterization and ultimate cloning of molecules expressed on the T cell surface. A major use of transformed cells has been the definition of the biochemistry of intracellular signaling during cell differentiation and/or activation. At present we have not determined whether the T cell clones described here are responsive to extracellular signals such as TCR ligation, which has been shown in mammalian T cells (e.g., reference 39) and in normal chicken T cells (22, 40) to induce changes in T cell physiology. In addition, however, the results demonstrated here provide the opportunity for further analysis of the mechanism(s) of cell transformation by the REV-T virus.

The REV-T(CSV) receptor is currently undefined. Increasing numbers of bursal cells or activated T cells absorbed increasing amounts of transforming activity from REV-T(CSV)-containing supernatants. This, taken together with the observation that ex vivo splenic T cells fail to absorb such transforming activity, demonstrates that transformation of chicken T cells with REV-T(CSV) requires virus adsorption to cell-specific and saturable receptor(s). REV-T(CSV) transforms both B and T cells in vivo (11, 29) and in vitro. There is no reason to postulate a priori that distinct receptors are used for viral adsorption to B and T cells, and so candidate molecules for the REV-T(CSV) receptor would therefore be expressed on the surface of at least subpopulations of B cells and activated T cells.

The expression of REV-T(CSV) receptors on the surface of ex vivo bone marrow T cells has not been determined directly due to the low numbers of T cells (~2%) in bone marrow suspensions. Consequently, while it is attractive to suggest that at least some bone marrow T cells in vivo express REV-T(CSV) receptors, it cannot formally be ruled out that culture of bone marrow T cells together with other leukocytes and/or stromal cells within the bone marrow population induced viral receptor expression. Such induction would by necessity need to be extremely rapid since the half-life of active REV-T(CSV) in tissue culture is ~4 h.

We have been unable to demonstrate REV-T(CSV)-mediated in vitro transformation of immediately ex vivo splenic T cells from normal chickens. However, exposure of ex vivo spleen cells from surgically bursectomized (B cell-deficient) chickens to REV-T(CSV) in vitro resulted in transformation of T cells with a distribution of phenotypes indistinguishable from those seen in the transformed bone marrow cultures shown in Fig. 3. This is analogous to the transformation of T cells in vivo with REV-T(REV-A) (18) and suggests the presence in normal spleen of a low frequency of T cells susceptible to REV-T transformation.

While T cells transformed from normal bone marrow grew for extended periods of time in vitro in the absence of any exogenous stimulus, T cells transformed from activated spleen cell populations grew for a maximum of 6 wk, typically for 3-5 wk, after transformation and could not be cloned. For the sake of this discussion we will refer to those bone marrow-derived T cells in which we have observed prolonged cell growth as being immortalized and distinguish this from T cell transformation, while appreciating that at this stage it is not possible to determine whether they will indeed grow indefinitely. For practical purposes, however, we have observed continued clonal growth for at least 4-6 mo, sufficient time to generate very large quantities of cloned cells.

We can therefore distinguish, based on the growth properties of T cells transformed from bone marrow as compared with activated spleen, T cell transformation from T cell immortalization. Since both T cell transformation in the short term and T cell immortalization require the target cell to express REV-T(CSV) receptors, parameters other than viral receptor expression must limit the long-term growth potential of REV-T(CSV)–transformed cells. Such parameters may also limit the transformation of B cells or ex vivo splenic T cells such that REV-T(CSV) receptor expression is not the only limit to target cell transformation. Consequently, in vitro activation of splenic T cells may lead not only to REV-T(CSV) receptor expression but also to other (intracellular) changes in T cell physiology required for transformation.

The transforming oncogene of REV-T(CSV) is v-*rel* (3, 4), the founding member of the rel family of cytosolic and/or nuclear proteins, which includes its cellular homologue, c-*rel*, as well as the NFκB complex proteins p50 and p65 (41–43). The v-*rel*-encoded protein $pp59^{v\text{-}rel}$ has been found in the cytosol, associated with other members of the rel family, notably $pp75^{c\text{-}rel}$ (44), as well as a protein of 40-kD, pp40 (45), a member of the avian IκB family (46) and higher molecular mass proteins (47), which include the p105 precursor of NFκB p50 (48). The molecular mechanism by which v-*rel* expression transforms target cells is currently unclear, but has been linked to its ability to form intracytoplasmic complexes with other proteins (49). There is evidence that v-*rel* functions as a transcriptional regulator (50, 51), although whether this function is mediated directly by the regulation of transcription by $pp59^{v\text{-}rel}$-containing complexes, or indirectly, by $pp59^{v\text{-}rel}$ sequestering normally active transcription regulating complexes, is unclear. Nonetheless, under either circumstance the oncogenic properties of v-*rel* likely depend on the endogenous expression and/or activation of other members of the rel protein family, possibly including NFκB. Mitogen activation of mammalian T cells leads to the rapid activation of NFκB (52). Therefore, the susceptibility of mitogen-activated chicken T cells to REV-T(CSV)–induced transformation may reflect not only induction of viral receptor expression, but activation of intracellular rel-related signal-transducing complexes.

The dissociation of transformation from immortalization further suggests that the retroviral integration and transcription of v-*rel*, driven by the retroviral LTR promoter required for transformation, is not sufficient for immortalization. Thus, intracellular factors other than v-*rel* expression are likely required to maintain the transformed state leading to cell immortalization. The difference in growth properties between bone marrow T cells and mitogen-activated splenic T cells demonstrates that independent of whether or not the bone marrow T cells are activated in vivo, they are nonetheless physiologically distinct from in vitro mitogen-activated splenic T cells. Therefore, it is possible that not only does the initial transformation of cells by v-*rel* require the coincident presence of active complexes containing rel-related proteins, but that the persistence of the transformed phenotype leading to immortalization requires that such complexes be constitutively active. The failure to isolate transformed and/or immortalized $CD4^+$ T cells from bone marrow, despite their presence in the ex vivo bone marrow population, further suggests that there are physiological differences between $CD4^+$ and $CD4^-$ (including but not restricted to $CD8^+$) T cells in the normal bone marrow. Whether this reflects differences in retroviral receptor expression or intracellular differences is not currently clear.

Short-term transformation of in vitro activated chicken T cells by exposure to REV-T(REV-A) in vitro has been described recently elsewhere (53). While it is difficult to judge from these results which populations of splenic T cells were transformed, growth of REV-T-infected splenic T cells was maintained in supernatants enriched for IL-2. It is quite possible therefore that the growth of the REV-T(CSV)–transformed T cells derived from activated spleen cells described here might be extended in the presence of appropriate cytokines. While this approach does not yield truly immortalized cells, dependent as they are on exogenous cytokines, it might provide a means of cloning and expanding selected populations of chicken T cells, since it is likely that the initial mitogen activation can be replaced by antigen-specific (or anti-TCR antibody) induced activation.

The efficiency of transformation of bone marrow cells (based on colony formation in soft agar; Table 1) suggests that at least 1 in 50 ex vivo bone marrow T cells can be transformed by REV-T(CSV). About 1 in 400 activated splenic T cells are transformed by REV-T(CSV), as judged by limiting dilution (Fig. 5). These frequencies compare very favorably with human T cell transformation by HTLV-I or HTLV-II, where efficiencies of transformation are considerably lower and frequently require that the target cells be cocultured with virus-producing cell lines (54). To date, rel-based constructs have not been effective in transforming mammalian cells. However, the results demonstrated here suggest that should this limitation be overcome, oncogenic forms of mammalian rel should provide a potent means of transforming mammalian T cells.

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