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E2A Deficiency Leads to Abnormalities in αβ T-Cell Development and to Rapid Development of T-Cell Lymphomas

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The E2A gene products, E12 and E47, are critical for proper early B-cell development and commitment to the B-cell lineage. Here we reveal a new role for E2A in T-lymphocyte development. Loss of E2A activity results in a partial block at the earliest stage of T-lineage development. This early T-cell phenotype precedes the development of a T-cell lymphoma which occurs between 3 and 9 months of age. The thymomas are monoclonal and highly malignant and display a cell surface phenotype similar to that of immature thymocytes. In addition, the thymomas generally express high levels of c-*myc*. As assayed by comparative genomic hybridization, each of the tumor populations analyzed showed a nonrandom gain of chromosome 15, which contains the c-*myc* gene. Taken together, the data suggest that the E2A gene products play a role early in thymocyte development that is similar to their function in B-lineage determination. Furthermore, the lack of E2A results in development of T-cell malignancies, and we propose that E2A inactivation is a common feature of a wide variety of human T-cell proliferative disorders, including those involving the E2A heterodimeric partners tal-1 and lyl-1.

The E2A gene encodes two basic helix-loop-helix (HLH) transcription factors, E12 and E47 (32). E12 and E47, members of the class I HLH proteins, are characterized by their broad expression pattern and their ability to bind DNA either as homodimers or as heterodimers with tissue-specific HLH proteins (9, 22, 33, 40, 43). Class I HLH proteins share several highly conserved domains. The HLH domain mediates homo- and/or heterodimerization, and the basic region constitutes the sequence-specific DNA binding domain (11, 22, 32, 50). In addition, two distinct domains located in the N-terminal portion of the class I HLH proteins have been shown to be required for transactivation (1, 26, 39).

E12 and E47 arise through differential splicing to the exon that encodes for the HLH domain. Within the HLH domain, their amino acid sequences differ by 20% (32). Both E12 and E47 have the ability to form heterodimers with class II HLH members, including the myogenic regulators (9, 23). However, E12 and E47 have distinct biochemical properties. E47 homodimers bind with high affinity to DNA, whereas an inhibitory domain present in E12 prevents those homodimers from high-affinity DNA binding (46).

E2A polypeptides bind to E-box sites present in a wide variety of tissue-specific enhancers, including the insulin, muscle creatine kinase, and immunoglobulin (Ig) intronic and 3′ enhancers (16, 22, 28, 32, 35, 38, 52). In B cells, it is homodimers of the E2A gene products that bind to E2-box sites present in the Ig enhancers (2, 34, 43). That E2A gene products play a crucial role in B lymphocyte development has been demonstrated recently by the generation of E2A-deficient mice and transgenic mice overexpressing Id1, an inhibitor of E2A (3, 47, 56). In the absence of E2A activity, even the earliest committed B-cell precursors are undetectable in the bone marrow (3, 47, 56). In addition, E2A-deficient mice lack Ig gene rearrangements (3).

In situ hybridization analysis revealed a high level of expression of E2A in the thymus (40). In addition, E-box binding sites have been identified in a number of T-cell-specific genes, including the CD4 enhancer, the CD4 silencer, and the T-cell receptor β enhancer (12, 42, 48). It has been shown that a heterodimer of HEB and an E12-related protein is capable of binding the E-box site in the CD4 enhancer, an element shown to be crucial for the activity of the enhancer (42). Furthermore, overexpression of Id blocked CD4 enhancer activity in T cells (42). The E-box site in the CD4 silencer has been shown to be important for the silencer activity, and the factor binding this site contains the same sequence specificity as the factor binding to the E box in the CD4 enhancer (12). Based on these studies, it is likely that the E2A gene products play a role T-cell development.

The studies described here reveal a new role for E2A proteins in T-lymphocyte differentiation. The absence of the E2A gene products leads to abnormalities in the earliest stages of αβ T-cell development and to dramatically reduced numbers of thymocytes. We propose a role for E2A gene products during thymocyte development that is similar to their function in B-lymphocyte differentiation. In addition, the E2A-deficient mice become prone to developing highly malignant T-cell lymphomas after 2 months of age. The lymphomas generally express high levels of c-*myc*, and each of the tumor DNAs analyzed showed a nonrandom gain of chromosome 15, which contains the c-*myc* gene. We propose a model in which E2A gene products are inactivated in human T-acute lymphoblastoid leukemias (T-ALLs), contributing to the development of lymphomas.

MATERIALS AND METHODS

Mice. The E2A- and E47-deficient mice were generated as described previously (3, 4). The original null mutant lines (a mixture of the 129/Ola and FVB/NJ

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backgrounds) were inbred four to five generations into the FVB/NJ background (Jackson Laboratory, Bar Harbor, Maine).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from thymocytes as described previously (2). Binding reactions were performed with 10 μg of nuclear extract and an oligonucleotide containing the μE5 E2-box sequence (2). For supershift assays, nuclear extracts were preincubated with the following monoclonal antibodies: 32.1 (anti-E47 [α-E47], 34.3 (α-E12), and 382.6 (α-E12/αHEB). All antibodies were generated at Pharmingen, San Diego, Calif.

Flow cytometric analysis. To analyze cell surface molecules, we used the following antibodies (all obtained from Pharmingen): α-CD4-phycoerythrin (RM4-5); α-CD8a-fluorescein isothiocyanate (FITC) (53-6.7); α-αβ TCR-FITC (H57-597); α-CD25-FITC (interleukin-2 [IL-2] receptor α chain; 7D4); and α-CD44-biotin (IM7). Staining of thymocytes has been described previously (3).

Propidium iodide staining. One million thymocytes were fixed in 70% ethanol for 30 min on ice. Cells were washed twice with phosphate-buffered saline, resuspended in 1 ml of phosphate-buffered saline containing 10 μg of propidium iodide, and analyzed on a FACScan.

Rearrangement Southern analysis. Genomic DNA was prepared from thymocytes by using DNAzol (Gibco, BRL). Ten micrograms of DNA was digested with *Eco*RI overnight, and samples were electrophoresed on an 0.8% agarose gel in 0.5× Tris borate buffer. Blots were probed with a 1.2-kb *Eco*RI-*Cla*I genomic fragment containing the Jβ2 locus.

Histology. Tissues were harvested from euthanized wild-type, E2A-deficient and E47-deficient mice and fixed in 1% paraformaldehyde. Tissues were processed and stained with hematoxylin and eosin at A Cut Above, San Diego, Calif. Light microscopy was performed at a magnification of ×150.

In vivo tumor induction. Thymocytes were prepared aseptically as described above from outwardly ill E2A and E47 mice and from a wild-type age-matched control. Cells from each tumor and from the control mouse (4.5×10^6 in each case) were injected intraperitoneally into three or four nude mice (N:NIH-bg-nu-xid BR background; Charles River Laboratories).

Northern analysis. Total RNA was prepared from thymocyte suspensions by using Trizol (Gibco, BRL). Fifteen micrograms of each sample was electrophoresed on a formaldehyde–1% agarose gel in morpholinepropanesulfonic acid-acetate buffer and then transferred to a Nytran membrane (Schleicher & Schuell) by capillary blotting. The blot was probed sequentially with cDNA fragments for c-Myc and β-actin. The relative amounts of c-*myc* RNA in tumor and normal thymus samples were quantitated by phosphorimager analysis, and these data were then normalized for RNA content in each sample, using the signal quantitated after probing for β-actin.

Comparative genomic hybridization. Comparative genomic hybridization was performed essentially as described previously (21). Wild-type thymus and tumor DNAs were labeled with fluorescein-12-dCTP and Texas red-5-dCTP. Normal metaphase chromosomes derived from the spleen were denatured and dehydrated by ethanol, hybridized at 37°C for 3 days, and washed. Slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) in an antifade solution. Profiles of fluorescence intensities of wild-type and tumor DNA signals, and the intensity ratio profiles, were calculated as described previously (37). Ratio profiles were normalized such that the average value was 1 for the whole genome.

RESULTS

Presence of E47 binding activity in thymus nuclear extract. It has been shown previously that E2A-HEB heterodimers bind to an E-box recognition element at a functionally important site in the CD4 enhancer (42). Additional E-box binding activity distinct from that of the E2A-HEB heterodimer has been detected in nuclear extracts from a Th2 cell line (D10), using the E-box site located in the CD4 silencer element (12). To assay for the presence of E12 or E47 binding activity in thymus nuclear extract, we performed EMSAs using the μE5 binding site and a panel of monoclonal antibodies specific for distinct members of the class I family of HLH proteins. The presence of an E-box binding complex containing both E47 and HEB is demonstrated by the significant decrease in complex formation seen upon addition of monoclonal antibodies to either of these proteins (Fig. 1, lanes 1, 2, and 4). However, the complex generated with thymus nuclear extract contains little to no E12 binding activity (Fig. 1, lane 3).

We next assayed for the presence of E-box binding activity in thymus extract prepared from an E47-deficient mouse. The binding complexes generated by E47-deficient thymus extracts are decreased to 35% of wild-type levels, and E47 binding activity is no longer detectable (Fig. 1, lanes 5 and 6). However, HEB binding activity is still present, thus demonstrating that in the thymus, E2A gene products are not required for expression

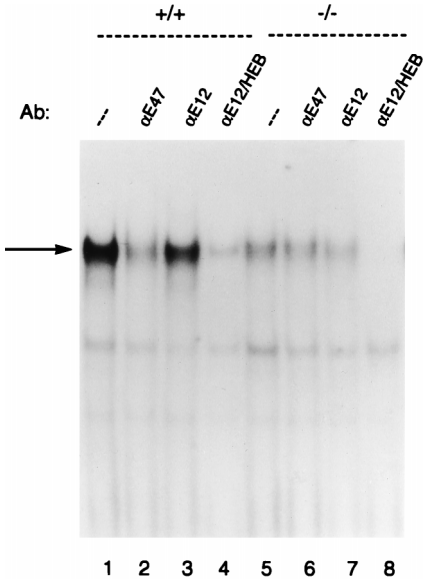


FIG. 1. Analysis of E2-box binding complexes in wild-type and knockout mice by EMSA of thymus nuclear extract from an E47-deficient mouse (−/−) and a wild-type littermate (+/+). Extracts were preincubated with monoclonal antibodies (Ab) against E47 (lanes 2 and 6) and E12 (lanes 3 and 7) or an antibody recognizing both E12 and HEB (lanes 4 and 8) before the addition of a μE5 oligonucleotide. The arrow at the left indicates the E2A- and HEB-containing complex.

of HEB or for DNA binding to an E2-box element by HEB homodimers (Fig. 1, lane 8).

Abnormalities in thymocyte development. To examine whether E2A proteins play a role in T-lineage development, thymocytes from E2A-deficient mice were analyzed by flow cytometry. E2A-deficient mice have, on average, fivefold fewer thymocytes than their wild-type and heterozygous littermates (Fig. 2A). Similarly, splenic T-cell numbers are reduced to 14 to 21% of wild-type numbers (Fig. 2A). Analysis of thymocyte phenotype by CD4 and CD8 surface staining revealed the presence of all thymocyte subpopulations. However, mice lacking E2A consistently showed significant decreases in the percentage of the $CD4^+$ $CD8^+$ population, with concomitant increases in the percentages of both $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ populations (Fig. 2B). Interestingly, the increase in the percentage of the $CD8^+$ thymocytes is always greater than that of the $CD4^+$ thymocytes, which results in a markedly different CD4/CD8 thymocyte ratio (1.8) in E2A-deficient mice compared to their wild-type littermates (4.4) (Fig. 2B and C).

In an E2A-deficient thymus, the most dramatic decreases in absolute cell numbers occur in the immature double-negative (DN) and double-positive (DP) populations. There are several possible explanations for this phenotype. The DP population of thymocytes is susceptible to apoptosis through negative selection (17, 49). Thus, it is conceivable that an E2A deficiency leads to increased levels of apoptosis. However, we have analyzed E2A-deficient thymuses by TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) staining and found no increase in the number of apoptotic cells compared to wild-type thymus (data not shown). Alternatively, there may be a developmental defect which results in the production of fewer numbers of DP cells. The DN stage of T-cell development can be divided into discrete stages based on the differential expression of the CD44 and CD25 cell surface molecules. The most immature cells are $CD44^{hi}$ $CD25^-$ and progress to a stage in which they gain

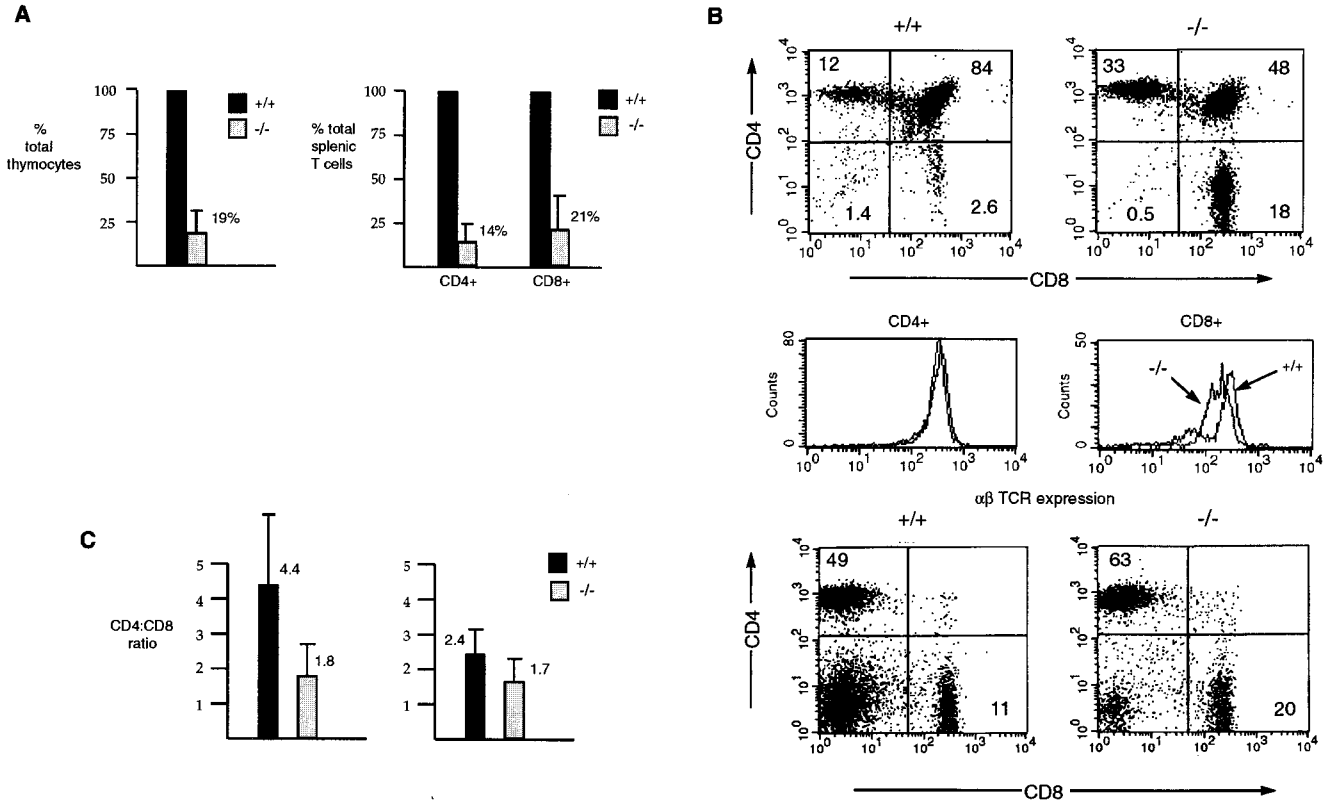
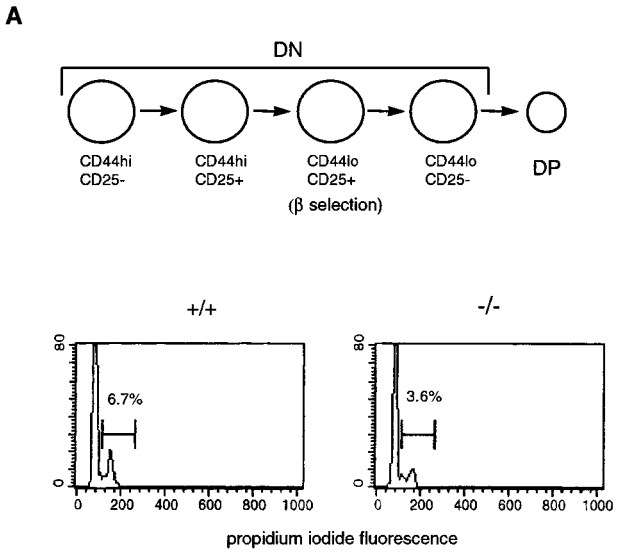


FIG. 2. Analysis of T-cell subsets from 4- to 8-week-old mice. (A) Average numbers of T cells from wild-type (+/+) and E2A-deficient (−/−) thymus (left graph) and spleen (right graph). Wild-type average number was set at 100%. E2A-deficient mice have significantly reduced numbers of thymic and splenic T cells. Total thymocyte numbers average 19% (±10%) of wild-type numbers ($n = 13$). $CD4^+$ splenocytes are decreased to 14% (±10%) of wild-type number, and $CD8^+$ splenocytes are decreased to 21% (±19%) of wild-type numbers ($n = 5$). (B) FACScan analysis of thymocytes (upper panel) and splenocytes (lower panel) from a wild-type mouse and an E2A-deficient mouse, using α-CD4 and α-CD8 antibodies. αβ TCR expression on the $CD8^+$ and $CD4^+$ populations is shown in the middle panel. In the thymus, the percentages of the single-positive populations are significantly higher in E2A-deficient mice. In all mice analyzed, the increase in the $CD8^+$ percentage was always higher than the increase in the $CD4^+$ percentage ($n = 13$). In addition, the $CD8^+$ population in the E2A-deficient mice displays lower cell surface expression of αβ TCR compared to $CD8^+$ thymocytes from a wild-type littermate. $CD4^+$ and DP thymocytes from E2A-deficient mice have approximately normal levels of surface TCR expression. Although the percentage of splenic T cells is increased in E2A-deficient mice (due to the absence of B cells), the total number is decreased significantly. (C) Average thymic (left panel) and splenic (right panel) CD4/CD8 ratios from wild-type and E2A-deficient mice ($n = 13$). The decrease in the CD4/CD8 ratio is more pronounced in the thymus.

CD25 expression ($CD44^{hi}$ $CD25^+$) (53). T lymphocytes of the next stage ($CD44^{lo}$ $CD25^+$) are in the process of β-chain selection (Fig. 3A) (13, 18, 24, 36). After production of a functional β chain, a DN thymocyte will undergo several rounds of proliferation before becoming a DP cell. We have analyzed E2A-deficient mice for defects in the DN stage of T-cell development by staining with α-CD44 and α-CD25 antibodies. Mice lacking E2A display increased percentages of the most immature DN cells ($CD44^{hi}$ $CD25^-$) and almost completely lack $CD25^+$ cells (12.5% versus 0.5%) (Fig. 3B). E2A-deficient mice also show a twofold decrease in the number of G_2/M-phase thymocytes, consistent with a partial block before the $CD44^{lo}$ $CD25^+$ stage (Fig. 3A). Thus, the E2A deficiency leads to a partial block at the earliest stage of thymocyte development.

Rapid development of T-cell lymphomas in E2A-deficient mice. The E2A null mutant mice display respiratory distress and ruffled coats beginning at approximately 3 months of age. All of the five mice monitored became moribund by 220 days (Fig. 4A). Upon necropsy, all of these mice displayed grossly enlarged thymuses. Histological examination of the thymuses revealed that the characteristic cortical and medullary structures were absent in the animals that developed tumors and were replaced by large lymphoblasts (Fig. 5A). These lymphoblasts often invade the spleen, abolishing its wild-type architecture (Fig. 5A). The lymph nodes, kidney, liver, and lung frequently showed evidence of metastasis (Fig. 5B and data not shown). We note that we have analyzed four other 3- to 4-month-old E2A-deficient mice which, although seemingly healthy, had developed thymomas. However, because most E2A null mutant mice die shortly after birth (3, 56), it was difficult to assess the frequency at which these tumors arise. More recently, we have generated an E47-deficient mutant mouse that expresses low levels of E12 (4). Unlike the E2A-deficient mice, over 50% of the E47 null mutants survive to maturity. We monitored 60 E47-deficient mice and found that between the ages of 76 to 230 days, 80% had become ill due to the development of thymomas, with 50% becoming ill by day 123 (Fig. 4A); 80% of the tumors identified in the moribund E2A- and E47-deficient mice displayed evidence of metastasis. Collectively, these data indicate that by 3 months of age, E2A-deficient mice are highly susceptible to developing a malignant T-cell lymphoma.

Tumors in E2A-deficient mice are monoclonal populations of $CD4^+$ $CD8^+$ T cells. Tumors isolated from the thymus, spleen, and lymph nodes were examined by flow cytometry to determine their cell surface phenotypes. The analyzed tumors consisted predominately of DP thymocytes (Fig. 4B). The



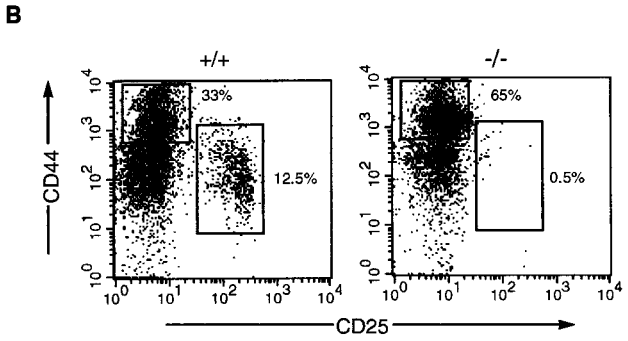
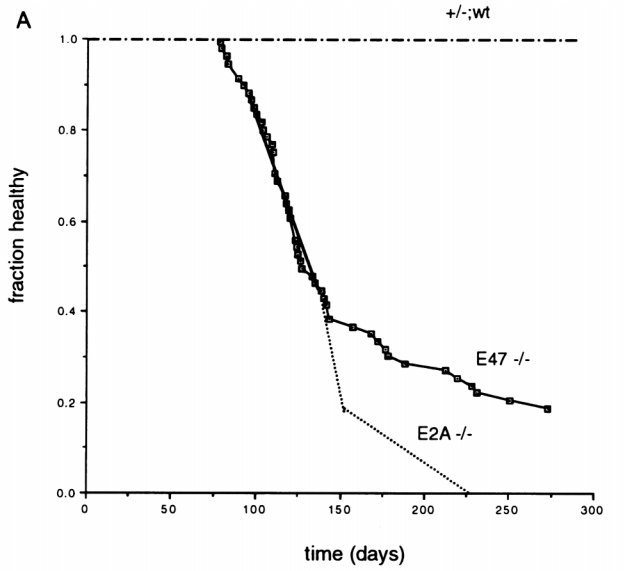


FIG. 3. Phenotypic analysis of the DN thymocyte subpopulation. (A) Schematic diagram of developmental stages within the thymocyte DN population. β-Chain selection occurs within the $CD44^{lo}$ $CD25^{+}$ population. Propidium iodide staining of total thymocytes as a measure of cycling cells is shown in the lower panel. The percentage of thymocytes in G_2/M is indicated; in E2A-deficient mice, the value averages 64% (±16%) of wild-type numbers ($n = 5$). (B) CD44 and CD25 staining of DN thymocytes. E2A-deficient mice almost completely lack $CD25^{+}$ DN thymocytes (0.5% versus 12.5%) and have an increased proportion of $CD44^{+}$ $CD25^{-}$ cells (65% versus 33%).

range of CD4 and CD8 expression was quite variable, but in almost all cases a fraction of the tumor population was $CD4^{+}$ $CD8^{+}$. In most cases, the lymphoblasts also expressed extremely high levels of the IL-2 receptor α chain (data not shown). To determine the clonality of the T-cell lymphomas, DNA was isolated from several tumors and analyzed for T-cell receptor (TCR) β-chain gene rearrangements. Southern blotting was performed with a Jβ2 probe to determine the Dβ-to-Jβ2 and Vβ-to-DJβ2 rearrangements on DNA derived from E2A and E47 null mutant thymomas. The T-cell tumors from both knockout mice display two predominate bands, indicating a clonal origin for the T-cell lymphomas (Fig. 4C). DNA isolated from a null mutant thymus at 1 month of age shows a heterogeneous mix of bands characteristic of a polyclonal population of cells (Fig. 4C). Thus, the absence of the E2A gene products can lead to the development of a monoclonal T-cell lymphoma expressing CD4, CD8, and in most cases the IL-2 receptor α chain.



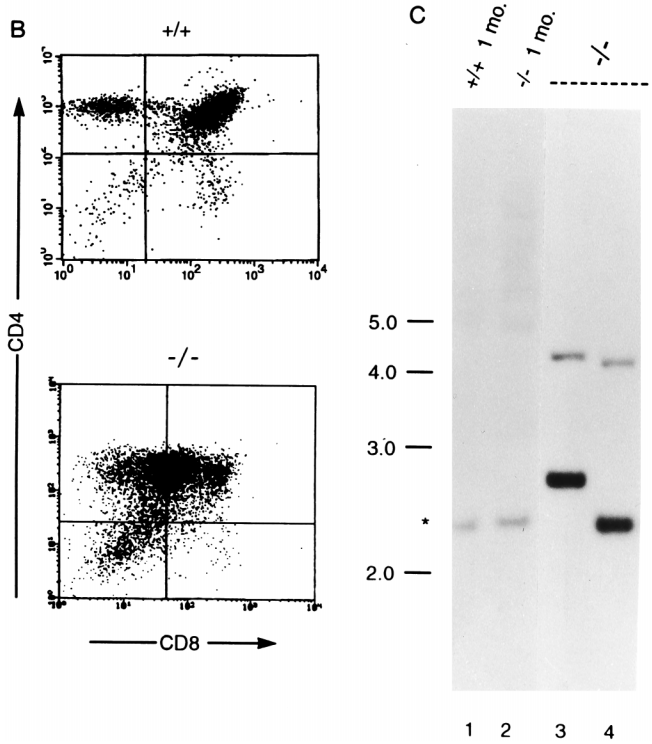


FIG. 4. E2A-deficient mice rapidly develop T-cell lymphomas. (A) Five E2A-deficient (E2A −/−) and 60 E47-deficient mice were observed for signs of disease. All of the five E2A-deficient mice showed signs of respiratory distress by 220 days, and 80% of the E47-deficient mice showed signs of respiratory distress by 230 days. All wild-type (wt) and heterozygous mice remained healthy within this time period. (B) CD8/CD4 profiles of thymocytes from an E2A-deficient mouse that had developed a thymoma and those from a wild-type littermate. (C) Southern blot analysis of D-Jβ2 and VDJβ2 rearrangements in genomic DNA from two different E2A null tumors (lanes 3 and 4). DNA was digested with *Eco*RI and probed with a Jβ2 probe. A wild-type thymus (lane 1) and a 1-month-old E47 null thymus (lane 2) display a heterogeneous population of bands indicative of a polyclonal population of cells. The germ line band is indicated by an asterisk. Positions of molecular weight standards are indicated in kilobases at the left.

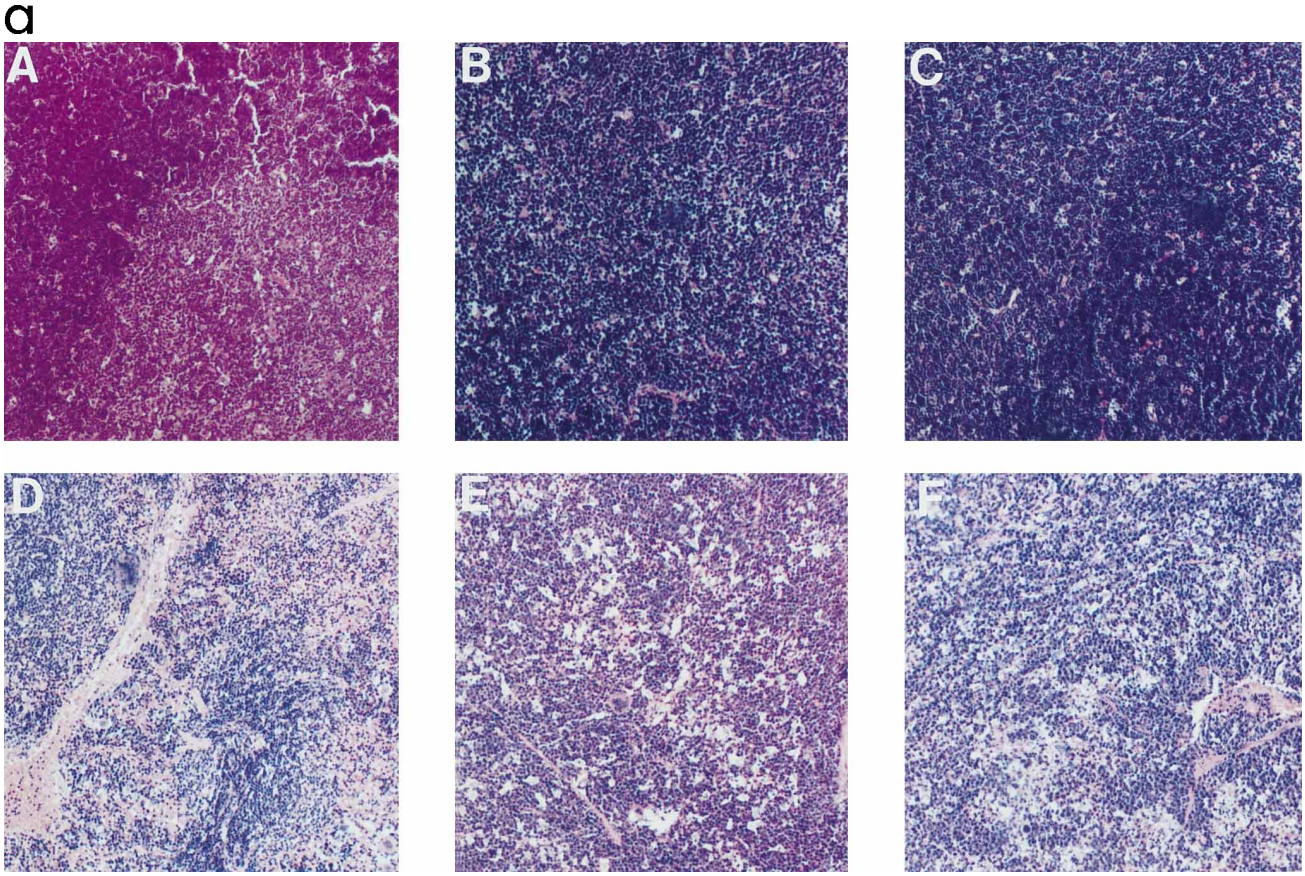


FIG. 5. Invasion of the lymphoid and nonlymphoid organs by lymphoblastic cells. (a) Hematoxylin-and-eosin-stained tissue sections from thymuses (upper panel) and spleens (lower panel) of 4-month-old wild-type (A and D), E2A-deficient (B and E), and E47-deficient (C and F) mice. Magnification, ×116. (b) Invasion of lung, kidney, and liver by lymphoblastic cells. Shown are hematoxylin-and-eosin-stained tissue sections of lungs (A to C), kidneys (D to F), and livers (G to I) from 4-month-old wild-type (A, D, and G), E2A-deficient (B, E, and H), and E47-deficient (C, F, and I) mice. Magnification, ×114.

Thymomas derived from E2A- and E47-deficient mice develop tumors in nude mice. To determine whether the T-cell tumors from E2A-deficient mice are able to invade organs of a host, T lymphocytes isolated from the thymuses of mutant mice with lymphoma were injected intraperitoneally into nude mice. By 3 weeks postinjection, the abdomens of the mice were greatly distended compared to those of the control injected mice (data not shown). By 3.5 weeks of age, when the first mice were sacrificed, the injected thymocytes had colonized the peritoneum and invaded the spleen and lymph nodes. Cells isolated from the affected organs had a cell surface phenotype similar to that of the injected cells, thus demonstrating the malignancy of the tumors isolated from E2A-deficient mice.

E2A deficiency leads to aberrant induction of c-*myc* transcription. The question raised is how the absence of E2A leads to the development of T-cell lymphomas. Pretumor E2A-deficient thymocytes do not display hyperproliferation, increased resistance to apoptosis, or increased levels of cell survival in vitro (5). To address further the underlying mechanism of tumor development in these mice, we examined whether the deficiency in E2A leads to the ectopic expression of proto-oncogenes. Interestingly, most tumors analyzed showed dramatic (between 10- and 17-fold) increases in the level of c-*myc* transcripts (Fig. 6). We do not detect significantly higher levels of c-*myc* transcripts in thymocytes derived from E2A-deficient mice in a pretumorous state, indicating that E2A does not directly regulate the expression of the c-*myc* gene (data not shown).

E2A-deficient tumors show chromosome abnormalities, including nonrandom gains of chromosome 15. To determine how the c-*myc* gene became activated, DNA derived from premalignant tumors was analyzed by Southern blotting and comparative genomic hybridization. We were unable to detect any genomic rearrangements of the c-*myc* locus by Southern blotting (5). Comparative genomic hybridization allows the detection of alterations in DNA copy number of total genomic DNA throughout the entire genome of tumor cells. This is accomplished by differential labeling of genomic DNA derived from wild-type and tumor cells, which are hybridized to metaphase spreads of wild-type mouse chromosomes (21). Chromosomal domains that show gains or losses can be identified as increases or decreases, respectively, in the fluorescence signal from the tumor DNA relative to that of wild-type DNA. We analyzed four tumor DNA samples, three from mice that did not display metastasis (one of which did not appear ill when sacrificed) and one from a mouse with a metastatic tumor. Interestingly, all tumors that were analyzed showed a nonrandom gain of chromosome 15, and two of the tumors also showed a gain of chromosome 14 (Fig. 7). It is interesting that chromosome 15 contains the c-*myc* proto-oncogene (25). Whether the presence of one extra copy of the c-*myc* proto-oncogene is sufficient for its elevated level of expression re-

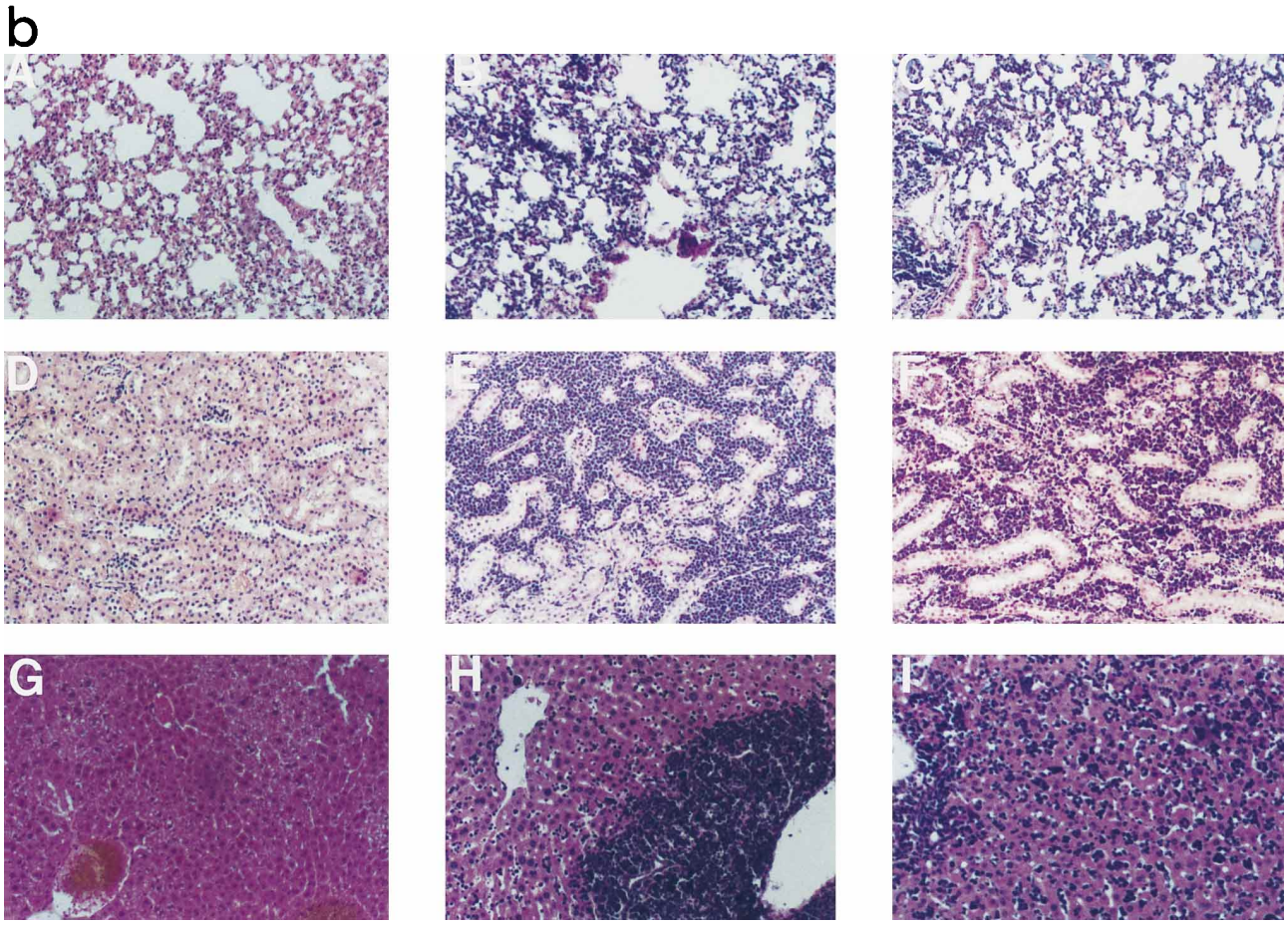


FIG. 5—*Continued.*

mains an open question, but it is interesting that the gain of one copy of chromosome 15 correlates with the activation of *myc* expression and the development of T-cell lymphomas.

DISCUSSION

Mice deficient for E2A were previously shown to be blocked at the earliest stages of B-cell development, prior to Ig gene rearrangements (3, 47, 56). The data described here reveal a new role for E2A proteins in T cells. E2A-deficient mice have dramatically reduced numbers of thymocytes, with the most significant alterations at the very earliest stages of thymocyte development. E47-deficient mice also display decreases in total thymocyte numbers, with an average three- to fourfold decrease compared to littermate controls. In addition, thymuses from E47-deficient mice are characterized by the same skewing of the subpopulations.

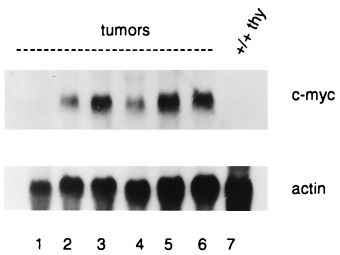


FIG. 6. Increased levels of c-*myc* RNA in E2A- and E47-deficient tumor samples, determined by Northern blot analysis of RNA derived from wild-type (+/+ thy) and tumor thymocytes (tumors), using c-Myc and β-actin as probes.

E2A-deficient mice display an increase in the percentage of the $CD44^+$ $CD25^-$ DN thymocytes, a population which contains cells not yet committed to the T-cell lineage (58). In addition, E2A-deficient mice almost completely lack $CD25^+$-committed DN thymocytes (58). The presence of E-box sites in the TCR β enhancer, and the importance of the enhancer for β rearrangement, suggests that E2A may be a critical regulator of TCR β rearrangement. However, the phenotypic defect identified in the DN population is clearly earlier than would be observed for defects in β rearrangement (30). Furthermore, the block is clearly prior to the block observed in RAG null mutant mice (31, 44).

In B lymphocytes, E2A proteins are required for the development of cells committed to the B lineage (3, 4, 47, 56). Specifically, B-lineage development in E2A-deficient mice is blocked at fraction A, an early stage of B-cell development characterized by Ig genes in the germ line configuration and the presence of cells not yet committed to the B lineage (4, 15, 41). E2A deficiency leads to a similar, although less complete, block during T-cell development. The absence of E2A results in an increase in the relative percentage of uncommitted T-cell precursors ($CD44^+$ $CD25^-$). This population of cells is not yet undergoing rearrangement and contains the T-cell receptor

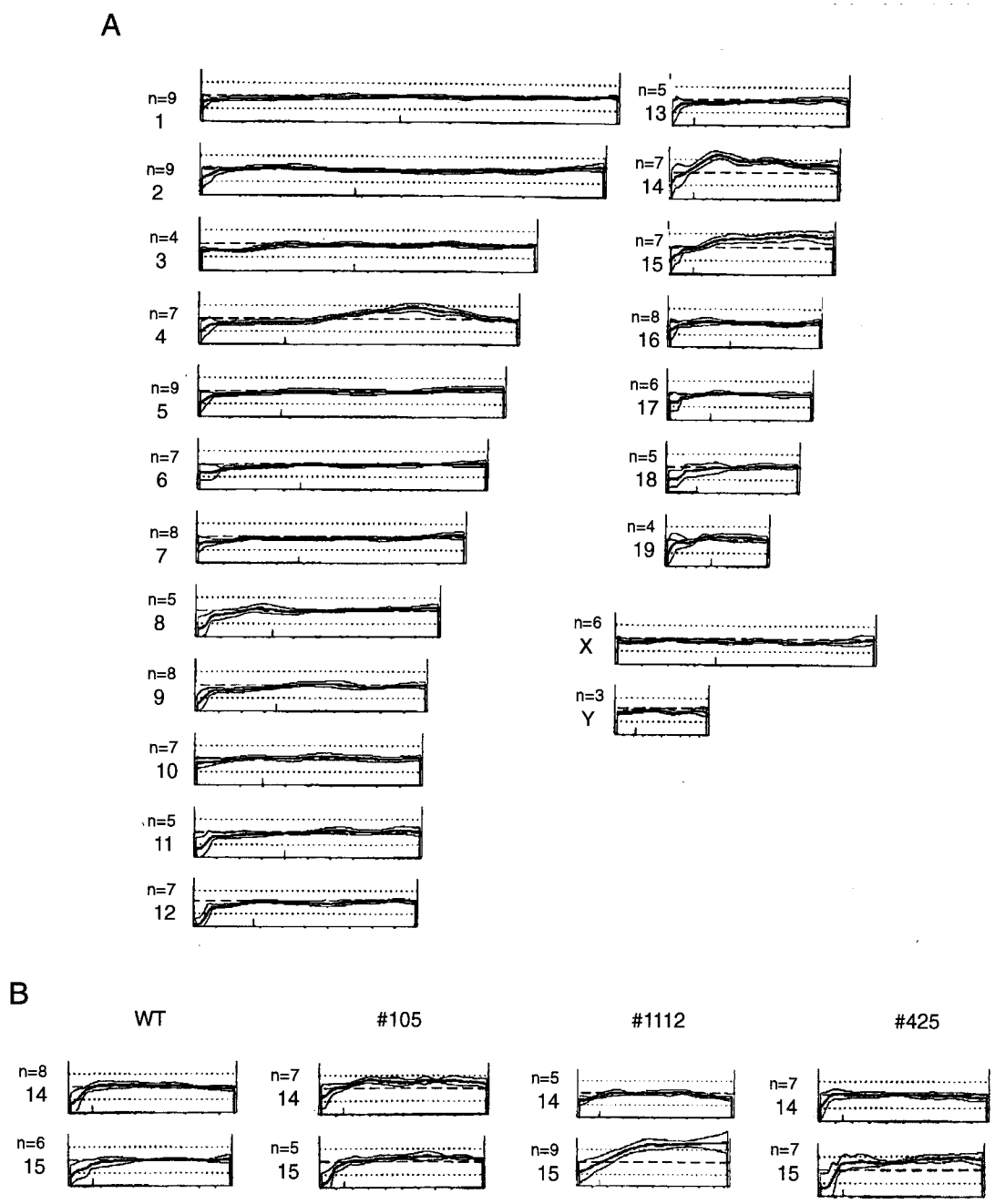


FIG. 7. Comparative genomic hybridization of four E2A-deficient tumor samples. (A) Fluorescence intensity profiles for chromosomes derived from tumor 205. The heavy lines indicate the average of profiles from several chromosomes as indicated by the *n* value. The thin lines represent ±1 standard deviation from the mean. The lengths of the diagrams are not proportional to the lengths of the chromosomes but represent normalized profiles. (B) Profiles of fluorescence intensity for chromosomes 14 and 15 derived from DNA isolated from three additional tumors (105, 1112, and 425) and a wild-type (WT) mouse.

genes in germ line configuration (58). In addition, E2A-deficient mice are characterized by a virtual absence of $CD25^+$ cells, the stage at which T-cell commitment takes place but TCR rearrangement has yet to be initiated. Thus, the defects in both the B and T lineages in E2A-deficient mice occur at almost identical developmental stages. However, unlike B-lineage development, which is completely blocked in E2A-deficient mice, T-lineage development is only compromised and mature T cells are generated at a low frequency. The most likely explanation for the leakiness of the T cell phenotype is the presence of HEB homodimers which may partially compensate for the loss of E2A.

How the absence of E2A contributes to the early defect in T-cell development is not completely clear. Thymocytes of the DN and DP stages of development are actively undergoing TCR gene rearrangements, a process that can result in the production of nonfunctional proteins or self-reactive TCRs. Cells which produce a self-reactive TCR molecule or fail to produce a functional TCR chain die by apoptosis (17, 49). We have analyzed thymuses from E2A-deficient mice by TUNEL

staining, a technique that allows the identification of apoptotic cells, and found no evidence of an increased level of apoptosis in mice lacking E2A (5). Furthermore, there were no significant differences in the survival of the DP thymocytes in vitro (5).

Within the DN stage of T-cell development, progression of uncommitted $CD44^+$ $CD25^-$ cells to the T-lineage-committed $CD25^+$ stage is important for reaching the DP stage of T-cell development. Others have identified two stromal factors, tumor necrosis factor alpha (TNF-α) and IL-1α, that are required for the progression of $CD44^+$ $CD25^-$ uncommitted thymic precursors to committed $CD25^+$ thymocytes (57). Addition of antibodies to both TNF-α and IL-1α in an in vitro culture system blocks the development of $CD25^+$ cells and results in the generation of dramatically reduced numbers of DP and single-positive thymocytes, a phenotype similar to that of the E2A-deficient mice (57). In addition, the reduction in thymocyte numbers and the decrease in the percentage of DP thymocytes observed in the E2A-deficient mice are similar to the thymic phenotype observed when mice are treated with a neutralizing antibody to IL-7 (14). Thus, it is possible that E2A acts downstream of either the TNF-α, IL-1α, or IL-7, signalling pathway. Alternatively, E2A may be required for the expression of these receptors.

At 4 months of age, approximately 50% of E2A-deficient mice developed T-cell lymphomas. The lymphomas represent highly malignant T cells with a CD4 and CD8 expression pattern that is similar to that of thymocytes in transition from the DP to single-positive stage of development. The tumors in E2A and E47 null mutant mice appear to be generated with similar frequencies. However, we note that the reduced levels of E12 in the E47 null mutant mice prevent us from determining whether it is solely the absence of E47 that leads to the development of the lymphomas. On the other hand, the development of tumors in both the E2A and E47 null mutant mice indicates that the underlying mechanism is caused by the mutations in the E2A gene and not by a mutation in the embryonic stem cells that were used to generate these mice.

E2A gene products are expressed in a number of different cell types (40), and so the question arises as to whether tumors, distinct from those derived in the thymus, develop in E2A-deficient mice. The T-cell lymphomas develop rapidly, and it is conceivable that the mice die before other cell types have a chance to develop into tumors. We have analyzed three E2A null mutant mice containing grossly enlarged sections of the gut (5). Whether these abnormalities are indeed the result of tumor development due to the E2A deficiency remains to be determined. It will be important to extend the studies described here and examine the potential role of E2A in tumor development of other cell types.

The mechanism of tumor development in E2A-deficient mice. Our findings raise the question of how the E2A deficiency results in the development of tumors. Thymocytes from E2A null mutant mice do not display hyperproliferation, nor do they show increased resistance to cell death induced by a number of stimuli both in vitro and in vivo (5). To address this issue further, we have examined whether the deficiency in E2A leads to the ectopic expression of proto-oncogenes. Most of the tumors examined showed dramatic (between 10- and 17-fold) increases in the level of c-*myc* transcripts. Ectopic expression of the *myc* gene has been shown previously to promote the development of T-cell lymphomas (8, 45). Thus, it is plausible that the increased levels of *myc* expression contribute to the development of the lymphoma. However, the question remains as to whether the E2A deficiency acts in concert with activated c-*myc* to promote development of the T-cell lymphomas or whether the absence of E2A results in chromosomal instability promoting the activation of the c-*myc* proto-oncogene.

The data also raise the question of whether the c-*myc* gene is directly activated by genetic alterations in the tumors. Analysis of several tumor DNAs by Southern blotting did not reveal genomic rearrangements of the c-*myc* locus (5). However, all tumors analyzed showed a nonrandom gain of chromosome 15, which contains the c-*myc* proto-oncogene (25). One of these tumors (tumor 1112) was derived from a mouse that did not exhibit outward signs of illness and as such is probably representative of a relatively early stage of tumor growth. Thus, the gain of chromosome 15 may be an early event in tumor development. Whether the presence of one extra copy of the c-*myc* proto-oncogene is sufficient to induce its elevated level of expression and consequently the development of lymphomas is an open question. It is conceivable that a dosage-sensitive repressor(s) of c-*myc* expression is titrated upon the gain of one extra copy of chromosome 15. On the other hand, it is possible that additional genetic alterations, such as the gains in chromosome 14 observed in some of the tumors, or other changes not detectable by using comparative genomic hybridization allowed the activation of the c-*myc* gene. Of course, the gain in chromosome 15 could also result in aberrant regulation of other genes in addition to c-*myc* that may be crucial for tumorigenesis. We should also note that the increase in c-*myc* expression may be due to events that cause cell cycle deregulation, as most of the tumors analyzed display an increase in the proportion of cycling cells relative to normal thymocytes (1a). Further experiments will be needed to address these issues.

Role of E2A in human T-ALL. One question that arises is whether E2A is also involved in human T-ALL. We have examined a number of human T-ALLs for the absence of E2A proteins but have not detected significant alterations in expression patterns (5). The most common genetic defect associated with human T-ALL is the activation of the *tal1* and *tal2* genes (6, 7, 10, 54). *tal1* and *tal2* are members of the HLH family and form heterodimers with E2A in both myeloid and erythroid cells (19, 55). However, the *tal* genes are normally not expressed in the thymus. The *tal* genes become activated and expressed in the thymus upon chromosomal translocation, which ultimately leads to the development of T-ALL. The *tal* gene products form heterodimers with E47, a protein which is normally a potent transactivator (1, 20, 26, 39). However, in the presence of *tal-1*, although DNA binding by E47 is still detectable, the transcriptional activity of E47 is abolished (20, 51). These data indicate that genes normally regulated by E47 homodimers cannot be activated in the presence of *tal-1* or *tal-2*. The results presented in this report suggest the possibility that E2A inactivation contributes, at least in part, to the development of a T-ALL (Fig. 8).

A second gene activated in T-ALL is *lyl-1*, which also encodes for an HLH protein. *lyl-1* is normally not expressed in the thymus but becomes transcriptionally activated upon translocation to the TCR β locus (27). Recently, *lyl-1* has been shown to associate with E2A to form a heterodimer which binds to sites distinct from those bound by the E47 homodimer (29). Thus, *lyl-1* may function as a dominant-negative mutant preventing the activation of E2A-responsive genes. Additionally, ectopic expression of *lyl-1* may redirect E2A to target genes normally not regulated by E2A (Fig. 8).

Taken together, these findings indicate that it is plausible that the inactivation of E2A target genes is an essential and common step toward the development of a number of T-cell malignancies.

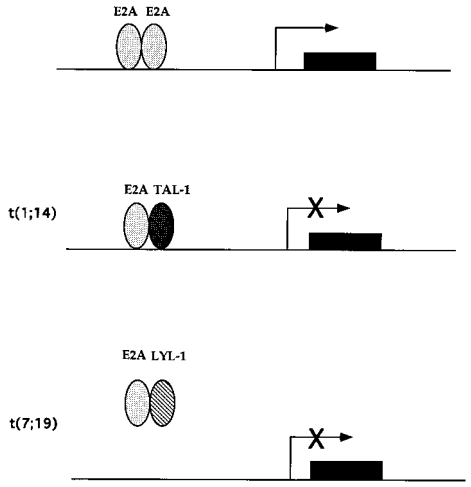


FIG. 8. Model proposing a role for E2A in a variety of human T-cell lymphomas.

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The first two authors contributed equally to this work.

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