Distinct transcriptional programs in thymocytes responding to T cell receptor, Notch, and positive selection signals

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T cell antigen receptor (TCR) signaling is necessary but not sufficient to promote the positive selection of $CD4^+CD8^+$ thymocytes into $CD4^+$ or $CD8^+$ mature T cells. Notch signaling has also been implicated as a potential regulator of both CD4/CD8 T cell development and TCR signaling. However, the relationship between positive selection, TCR signaling, and Notch remains unclear. Here we use DNA microarray analysis to compare gene expression changes in $CD4^+CD8^+$ double-positive thymocytes undergoing positive selection, TCR stimulation, and Notch activation. We find that the genes induced during positive selection can be resolved into two distinct sets. One set, which we term "TCR-induced," is also induced by *in vitro* TCR stimulation and contains a large proportion of transcription factors. A second set, which we term "positive-selection-induced," is not induced by *in vitro* TCR simulation and contains a large proportion of genes involved in signal transduction pathways. Genes induced by Notch activity overlap substantially with genes induced during positive selection. We also find that Notch activity potentiates the effects of TCR stimulation on gene expression. These results help to identify TCR- and positive-selection-specific transcriptional events and help to clarify the relationship between positive selection and Notch.

microarray | gene expression profile | negative selection

The outcome of thymic development is a mature T cell population that is self-tolerant and able to recognize foreign antigen with self-MHC proteins and in which each cell has chosen a T cell lineage appropriate for its T cell antigen receptor (TCR) specificity. Distinct signaling pathways operate in developing thymocytes to achieve this outcome, with the most important signals induced by the TCR. Precursor $CD4^+CD8^+$ double-positive (DP) thymocytes whose TCR recognizes self-peptide–MHC complexes with high affinity are negatively selected to eliminate potentially autoreactive T cells. In contrast, potentially useful thymocytes with moderate affinity for self-peptide–MHC are positively selected (reviewed in ref. 1). Positive selection toward either the CD4 or CD8 lineage depends on TCR specificity for the class of MHC. Recognition of MHC class I during positive selection leads to CD8 development, whereas recognition of MHC class II leads to CD4 development. It has been suggested that TCR signals promoting CD4 versus CD8 development differ in both strength and duration. How these TCR-signaling differences are interpreted at the transcriptional level remains to be determined.

In addition to TCR-induced signals, other signals provided by the thymic stromal environment are indispensable for T cell-positive selection. One contributing factor may be Notch, a receptor with conserved function in cell-fate determination. Expression of constitutively active Notch [Notch intracellular (NotchIC)] in DP thymocytes has dramatic effects on CD4 and CD8 T cell maturation, which have been interpreted by different groups as either effects on CD4 versus CD8 lineage commitment (2–4), promotion of thymocyte survival (5, 6), or dampening of TCR signals (7). It has also been proposed that Notch activity might promote CD8 T cell development by down-modulating TCR signals (7), thus linking Notch signaling to the quantitative TCR-signaling model for CD4 versus CD8 lineage commitment. Although thymocyte-specific deletion of the Notch1 gene does not perturb positive selection (8), other means of disrupting Notch signaling can block CD8 development (9, 10), suggesting that multiple Notch family members might participate in positive selection.

The signaling pathways that contribute to T cell maturation ultimately lead to changes in gene expression, and analysis of the transcriptional programs induced by these signaling pathways is crucial to our understanding of thymic development. DNA microarray technology has made it possible to monitor global changes in gene expression, thus opening the way to addressing these important issues. Several groups have used microarray analysis to examine gene expression in thymocytes undergoing TCR signaling (11–13). Although this microarray analysis represents an important first step to analyzing gene expression changes on TCR signaling in thymocytes, the relationship between the gene expression programs induced by positive selection, TCR signaling, and Notch signaling has yet to be addressed.

To investigate the individual and combined contributions of TCR and Notch to positive selection, we compared the transcriptional programs of thymocytes undergoing positive selection to the transcriptional programs induced by TCR or Notch alone. Using cluster analysis to identify sets of genes with related expression patterns, we identified a group of genes that are induced during positive selection but not by *in vitro* TCR stimulation. These positive-selection-specific genes are enriched for genes that function in signal transduction processes. In contrast, the TCR-induced genes contain a high proportion of transcription factors. Substantial overlap occurred between gene expression induced by Notch signaling and positive selection, suggesting that Notch signaling might contribute to thymocyte maturation. In contrast to previous reports, we found that Notch synergizes with TCR to induce more rapid and robust transcriptional regulation of TCR-induced and -repressed genes.

Materials and Methods

Mice. C57BL/6 (B6), $MHC^{-/-}$ (C57BL/6 β2m, I-$Ab^{-/-}$ I-E null, Taconic Farms), F5 TCR transgenic (tg) C57BL/6 (D. Kioussis, National Institute of Medical Research, London; ref. 14), P14 TCR tg C57BL/10 $Rag2^{-/-}$ (Taconic Farms), 5CC7 TCR tg C57BL/10.A $Rag2^{-/-}$ (Taconic Farms), AND TCR tg C57BL/10 (The Jackson Laboratory), and NotchIC tg (4) mice were maintained in the University of California, Berkeley mouse facility. Mice, 4–12 weeks of age, were used for analyses.

Thymocyte Isolation. Thymocytes from F5 and P14 TCR tg mice were incubated with anti-CD4 beads. Thymocytes from 5CC7 and AND TCR tg were incubated with anti-CD8 beads. Positive frac-

Abbreviations: TCR, T cell antigen receptor; DP, $CD4^+CD8^+$ double positive; tg, transgenic; NotchIC, Notch intracellular; SAM, statistical analysis of microarrays.

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tions were purified by AutoMacs magnetic bead separation (Miltenyi Biotec, Auburn, CA). Populations were determined to be 90–99% pure.

TCR Stimulation. $MHC^{-/-}$or NotchIC tg $MHC^{-/-}$ thymocytes (10^8/30 ml) were stimulated in plates coated with anti-TCRβ (5 μg/ml) and anti-CD2 (5 μg/ml) (BD PharMingen) for 2, 4, 8, or 16 h. A fraction of cells was analyzed for CD4 and CD8 down-regulation and CD5 and CD69 up-regulation. Lymph node T cells were purified to >95% purity on CD3+ T Cell Enrichment Columns (R&D Systems) and then incubated in media or as above for 8 h.

Microarray. Total RNA was isolated by using TRIzol (Invitrogen). RNA (10–20 μg) was labeled as described at www.microarrays.org with slight modifications (see *Supporting Methods*, which is published as supporting information on the PNAS web site). Labeled samples were hybridized overnight at 50°C on custom microarray slides described in ref. 15.

Significantly changed genes were identified by using statistical analysis of microarrays (SAM) (16). For each clone, individual $\log_2$ ratios from duplicate spots and replicate experiments were input into SAM to determine a "*d*" significance value. This value reflects the magnitude of change versus the variance between duplicate/replicate spots. The *d* values are then ranked to generate a list of significantly induced and repressed genes. We further analyzed genes with an absolute *d* value of one or greater and an absolute fold change of at least two. Genes that met these criteria were verified to have a low covariance.

Significant genes from select test populations of interest were compiled. Mean expression changes for genes and thymocyte populations of interest were tabulated with GENE TRAFFIC 4.2 and filtered to exclude genes detected in <80% of selected thymocyte populations. Each table was clustered with CLUSTER 2.12 by using uncentered average-linkage hierarchical clustering and visualized with TREEVIEW (17).

Flow Cytometry. Thymocytes were stained with combinations of anti-CD2, -CD4, -CD5, -CD8, -CD69, and -TCRβ conjugated to FITC, phycoerythrin, or phycoerythrin-Cy5 (BD PharMingen and eBioscience, San Diego) and anti-CD4 and -CD8 conjugated to phycoerythrin-Texas red (Caltag, Burlingame, CA). Cells were processed on a Coulter Epics XL-MCL and analyzed with FLOWJO software (Tree Star, Ashland, OR).

Results and Discussion

Global Analysis of Gene Expression Profiles in $CD4^+CD8^+$ Thymocytes. We used DNA microarray analysis to compare the transcriptional programs induced by TCR signaling, positive selection, and Notch signaling. We used $CD4^+CD8^+$ thymocytes from $MHC^{-/-}$ mice as a reference sample, because they represent resting precursors that have not received a TCR signal. To identify positive-selection-dependent transcriptional events, we used sorted $CD4^+CD8^+$ thymocytes from positively selecting TCR tg mice. To identify early TCR-responsive transcripts, we used $MHC^{-/-}$ thymocytes that had been stimulated *in vitro* by using plate-bound anti-TCR antibodies. To identify Notch-dependent transcription we used $CD4^+CD8^+$ from $MHC^{-/-}$ thymocytes expressing a constitutively activated Notch transgene (NotchIC).

The existing microarray libraries (e.g., RIKEN and NIA) contain mostly EST clones of unknown function and few immunology-related genes. We thus used custom microarray slides containing ≈2,700 handpicked sequence-verified cDNA clones spotted in duplicate. These genes primarily represent genes of known function and are enriched for genes with relevance to immune function. They include those involved in transcription, signal transduction, apoptosis, notch signaling, interleukins, cytokines, and many clusters of differentiation genes (Fig. 3, which is published as supporting information on the PNAS web site). Test and reference RNAs labeled with Cy3 and Cy5 dyes were hybridized to the custom slides. Each hybridization was repeated at least three times, including one in which the Cy labels were swapped between reference and test samples. Thus, for each condition, at least six measurements were done for each clone. The $\log_2$-fold difference between test and reference samples was calculated for each spot, and the data are reported as the mean difference for each gene for each condition examined.

Of the 2,700 clones, we focused our analysis on 177 genes whose expression varied significantly in at least one of the conditions examined. To generate this list, we subjected the data for each test sample to SAM (16). SAM calculates significance by evaluating the magnitude of the fold difference against the scatter between replicate hybridizations. By using this method, a clone with a mean $\log_2$-fold difference of only one (2-fold change) is significant if scatter is very small.

To resolve the 177 differentially expressed genes into categories based on their expression patterns, we used a hierarchical clustering algorithm (Fig. 1 and Fig. 4, which is published as supporting information on the PNAS web site). Genes with similar expression patterns are grouped together, and the tree diagram displayed on the left in Fig. 1 represents the relative similarity in expression pattern between different genes. When analyzed in this way, several general features are apparent. First, whereas a set of genes is induced by both TCR signaling and positive selection (cluster 2), a large group of genes (cluster 1) is induced selectively by positive selection and not by *in vitro* TCR stimulation. In contrast, repressed genes (cluster 3) tend to be repressed by both TCR and positive selection signals. In general, genes induced or repressed by Notch signaling were also induced or repressed by TCR and positive selection. However, a set of genes also was exclusively induced by Notch signaling (cluster 4). Very few genes showed expression differences when comparing $MHC^{-/-}$ thymocytes with unfractionated wild-type thymocytes. This outcome was expected because the majority of thymocytes in wild-type mice are probably not receiving positive-selection signals. In addition, control hybridizations in which wild-type thymocytes were compared to themselves showed virtually no expression differences, confirming the reliability of the data.

Genes Induced During Positive Selection. One large category representing 44 of the 177 differentially expressed genes are genes induced during positive selection but not by TCR signaling. This category includes many genes previously described to be induced during positive selection, such as CD53, IL-6Rα, and schlafen 1 (18–20), and some genes whose expression pattern during positive selection has not been previously characterized, such as semaphorin 4A, PKC-θ, and the serine protease inhibitor, serpin1b. This cluster may include genes that require thymic stromal cell factors in addition to TCR signaling for their induction. Alternatively, some of these genes may be induced late after TCR stimulation. Positive selection takes days to complete. DP thymocytes from positive selecting TCR tg mice have been reported to have a lifespan of 1–3 days (21, 22). Therefore, it is likely that the DP thymocytes from positive selecting TCR tg mice represent both early and late responses to TCR triggering. In contrast, we limited our *in vitro* TCR stimulation to 16 h because of the large amount of apoptosis found in cultures at later time points. Indeed, two of the genes within cluster 1, CCR7 and CD53, have been previously shown to be induced slowly after TCR stimulation (19, 23). Thus, the set of genes induced during positive selection but not by *in vitro* TCR triggering may reflect both stromal cell-dependent events and later events associated with TCR-dependent signaling.

One striking feature of this positive selection cluster is the heavy representation of genes involved in signal transduction. Nineteen of forty-four genes (43%) in this cluster correspond to signal transduction components in comparison with 29% in the TCR-induced

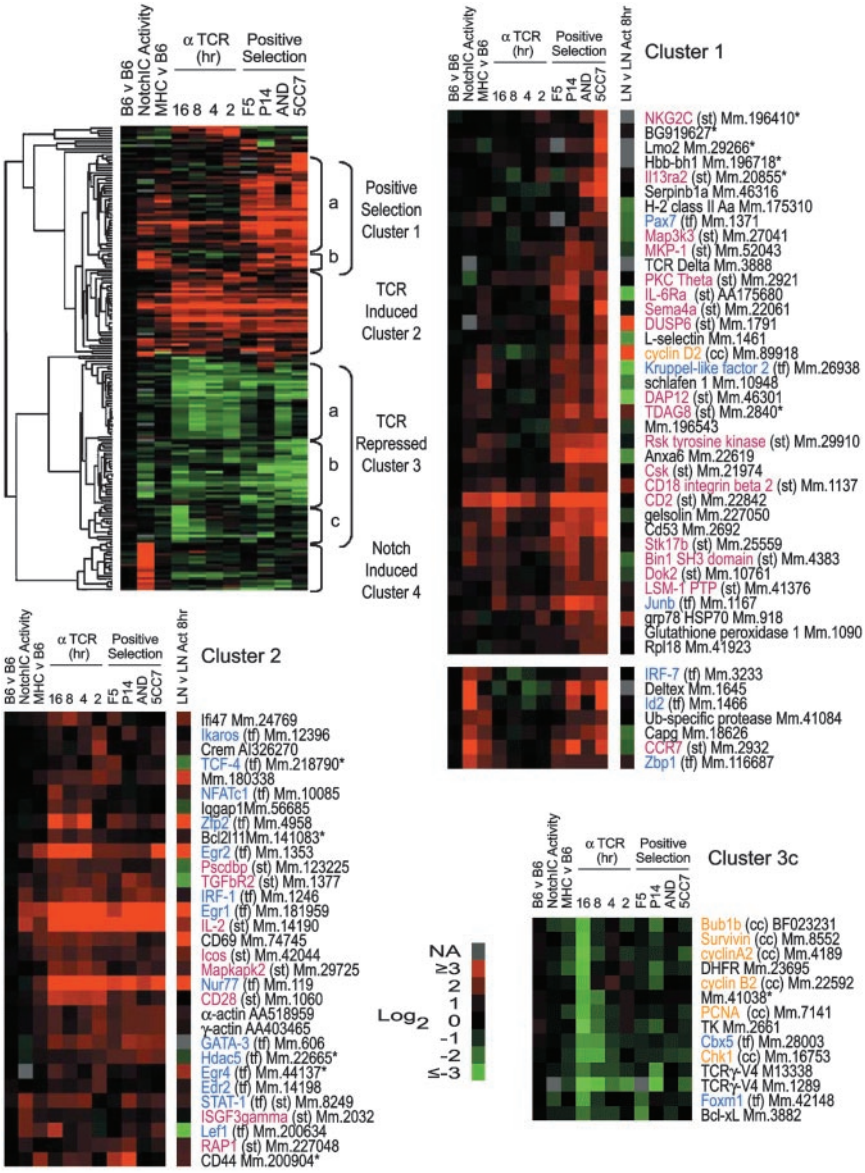

Fig. 1. Gene expression differences in thymocytes in response to positive selection, Notch activity, or TCR stimulation. Whole wild-type B6 thymocytes, MHC$^{-/-}$ thymocytes stimulated through TCR, or CD4^{+}CD8^{+} (DP) thymocytes from 5CC7, AND, F5, or P14 TCR or NotchIC tg mice were compared with a reference population with microarray slides spotted with ≈2,700 immunologically important probes. Sorted DP MHC$^{-/-}$ thymocytes served as reference for all DP test populations. Unfractionated MHC$^{-/-}$ thymocytes served as reference for all other test populations, unless specified otherwise. One hundred seventy-seven genes were identified to have statistically significant expression changes and clustered by using CLUSTER 2.12. Rows correspond to individual genes, and columns correspond to test thymocyte populations. Genes with similar expression patterns cluster together, and the tree diagram displayed on the left represents the relative similarity in expression pattern between genes. Four major expression clusters were apparent: genes induced during selection (1a and 1b), induced by TCR stimulation (2), repressed by TCR stimulation (3a, 3b, and 3c), and induced by NotchIC activity (4). Gene identities including UNIGENE V.116 numbers are listed with the following functional identifiers: st, signal transduction (purple type), tf, transcription factor (blue type); cc, cell cycle (orange type). Gene expression changes for lymph node T cells *in vitro* TCR-stimulated for 8 h compared with unstimulated T cells are displayed alongside clusters 1 and 2. Genes marked with * had low hybridization signal or high covariance in some thymocyte populations. Confirmation of individual expression patterns should be performed before further investigating individual genes. For data values, see Tables 2 and 3, which are published as supporting information on the PNAS web site.

cluster and 27% in the total list of regulated genes (Table 1, which is published as supporting information on the PNAS web site). Some of these genes have been previously implicated in the TCR-signaling pathway, such as PKC-θ and JunB. Others are part of distinct signaling pathways, such as the chemokine receptor CCR7 and Semaphorin 4a. This cluster also includes protein kinases, including MAP3K3 and Rsk, and protein phosphatases, including MKP1 and LSM-1. In contrast, transcription factors are relatively underrepresented in this cluster [6 of 44 (14%) compared with 45% in the TCR-induced cluster 2]. This finding suggests that the later stromal cell-dependent phase of positive selection involves modulation of signal transduction pathways in contrast to the early TCR-driven stage of positive selection, which involves modification of the transcriptosome of DP thymocytes. Signal transduction proteins are sharply underrepresented among repressed genes (Table 1, cluster 3). This underrepresentation suggests that thymocytes modify their signal transduction pathways by gene induction rather than by gene repression. Induction of genes encoding repressors of the pathway may provide for a more rapid down-modulation of signaling pathways than transcriptional repression, given the time required for protein turnover.

Positive-selection-induced genes can be further subdivided into genes that are also strongly induced by Notch signaling (cluster 1b) and those that are not (cluster 1a). The Notch-induced cluster contains a previously described target of Notch signaling, deltex (5), and other genes that to our knowledge have not been previously described as Notch targets, including the HLH transcription factor inhibitor Id2 and the chemokine receptor CCR7.

Genes Induced in Response to TCR Signals. A set of 31 genes was found to be induced both by *in vitro* TCR stimulation of MHC$^{-/-}$ DP thymocytes and by positive selection (Fig. 1, cluster 2). This set includes many genes previously shown to be inducible by TCR signaling in DP thymocytes, such as Nur77, GATA-3, and Egr-1 and -2 (24–27). This set of TCR-inducible genes contains a high proportion of transcription factors [14 of 31 (45%) compared with 19% in the 177 regulated genes (Table 1)]. This finding suggests that one early consequence of TCR signaling in the thymus is a reshaping of the configuration of transcription factors present in DP thymocytes.

Although both thymocytes and mature T cells are responsive to TCR signaling, their cellular responses are quite distinct. TCR

signaling induces either survival or apoptosis in thymocytes, whereas it induces proliferation and effector function generation in mature T cells. To compare the transcriptional program of mature T cells and DP thymocytes, we performed microarray analysis on mature T cells stimulated for 8 h under identical conditions that were used to stimulate DP thymocytes. We find that stimulation of mature T cells leads to significant changes in ≈160 of the 2,700 clones analyzed (as determined by SAM), similar to the number of genes found to be regulated by TCR stimulation of DP thymocytes. In addition, the average magnitude of expression changes was similar between mature T cells and DP thymocytes (4.4-fold average induction and 2.6-fold average repression compared with 3.2-fold average induction and 2.0-fold average repression for DP thymocytes). In addition, many genes whose expression was induced in DP thymocytes were also induced by TCR stimulation of mature T cells (Fig. 1, cluster 2). In contrast, very little correlation exists with the genes selectively induced by positive selection signals (Fig. 1, clusters 1a and 1b). These results are consistent with an earlier study comparing gene expression changes in TCR-stimulated T cells and thymocytes (11) and suggest that the early transcriptional response of DP thymocytes to TCR triggering is similar in magnitude and partially overlaps with the response of mature T cells.

In addition to the genes whose expression were previously shown to be regulated by TCR signaling in thymocytes, we found others whose induction by TCR was unexpected. Perhaps the most surprising is IL-2. IL-2 is a classic target of TCR signaling in mature T cells and has also been shown to be inducible in DP and single positive thymocytes. However, earlier studies have indicated that IL-2 is very poorly induced in DP thymocytes, possibly because of a failure to induce AP-1 transcriptional activity (28, 29). The strong induction of IL-2 seen in our study is consistent with indications that a subset of $CD69^+$ DP expresses IL-2 (30, 31) and suggests that the *in vitro* TCR stimulation conditions used here might lead to some AP-1 induction. It is also intriguing that several other genes related to cytokine signaling are found within the cluster of TCR-induced genes, including STAT-1, a signal transducer and transcription factor downstream of IFN-γ; IRF-1, an IFN regulatory family transcription factor implicated in CD8 T cell development (32); and ifi-47, an IFN-inducible protein. In addition, IL-6Rα transcripts are up-regulated in DP thymocytes from positive selecting TCR tg mice (Fig. 1, cluster 1a). As yet, no role for cytokine signaling in DP thymocytes has been firmly established, although the indications are that cytokine signaling might promote CD8 T cell development (33, 34).

The *in vitro* TCR stimulation conditions used here lead to massive thymocyte apoptosis by 24 h. Thus, these conditions appear to mimic negative selection signals. In this regard, it is interesting that virtually all the TCR-induced genes are also found to be up-regulated in positively selecting thymocytes. Two notable examples are Nur77 and Nor-1, transcription factors previously implicated in negative selection. Nur77, in particular, was shown to be highly expressed in negatively selecting thymocytes (35, 36). The induction of Nur77 and Nor-1 shown here in positively selecting thymocytes is unlikely to reflect a functional role for these proteins, because a dominant negative form of Nur77 blocks negative but not positive selection. The absence of a set of "negative-selection-specific genes" in our analysis may indicate that the early stages of positive and negative selection involve common sets of transcriptional targets. An alternative explanation is that some thymocytes within our positive selecting samples are actually undergoing negative selection. Indeed, other studies have provided evidence for mixed positive and negative selection in certain TCR tg mice (37, 38).

Genes Repressed in Response to TCR or Positive-Selection Signals. Sixty-nine of the 177 regulated genes were repressed after TCR stimulation. These genes include many genes previously shown to be down-regulated in DP thymocytes by TCR or positive selection, such as survivin, rag2, TdT, and RORγt (39–42). In general, a good correlation occurred between genes repressed by *in vitro* TCR stimulation and genes repressed during positive selection. This result implies that the early TCR response involves both gene induction and repression in contrast to the later stromal cell-dependent phases of positive selection that overwhelmingly involve gene induction (Fig. 1, cluster 1). However, cluster analysis allows us to separate the repressed genes into three categories, which differ in the degree to which they are repressed under *in vitro* stimulation conditions versus positive-selection conditions. The largest subgroup of genes was equivalently repressed in both TCR-stimulated $MHC^{-/-}$ thymocytes and TCR tg thymocytes (Fig. 1, cluster 3a). Genes in this cluster include the CD4 coreceptor, the orphan nuclear receptor RORγt, the transcription factor Snai2, and the ephrin receptor ephRB3. A second subgroup contains genes that are repressed to a greater extent in TCR tg thymocytes than in TCR-stimulated $MHC^{-/-}$ thymocytes (Fig. 1, cluster 3b). This cluster includes the coreceptor CD8β, the recombinase-activating gene rag2, the nucleotide transferase TdT, and the negative regulator of TCR signaling c-cbl. As with CD4, CD8β down-regulation occurs in response to *in vitro* TCR stimulation and in class I- and class II-restricted positive selecting thymocytes. This finding is consistent with early reports indicating that CD4 and CD8 are coordinately down-regulated during the early phase of positive selection (43, 44). This down-regulation of coreceptor mRNA likely reflects an immediate response to TCR triggering that is distinct from the lineage-specific repression of CD4 and CD8 genes that ultimately occurs after positive selection.

A small third subgroup contains genes that are strongly repressed after 16 h of *in vitro* TCR stimulation and weakly repressed under other conditions (Fig. 1, cluster 3c). This cluster contains an unusually high proportion of genes involved in cell-cycle regulation [6 of 14 (42%), Table 1], most of which are cell-cycle proteins that act at the replication and mitotic stages of the cell cycle. In general, cell-cycle proteins are not expressed in G_0 cells. For example, peripheral resting T cells do not express G_1 kinase or proteins involved in S and G_2/M phases of the cell cycle, but these proteins are up-regulated on stimulation through the TCR (45). Although the majority of DP thymocytes are not dividing, their expression of cell-cycle proteins is not typical of G_0 cells, and it has been suggested that DP thymocytes are actually proceeding slowly through the cell cycle (46). Unlike mature T cells, which proliferate in response to TCR stimulation, DP thymocytes either undergo apoptosis (negative selection) or differentiation without proliferation (positive selection) in response to TCR signals. It is tempting to speculate that the repression of cell-cycle promoters observed here contributes to the failure of DP thymocytes to proliferate in response to TCR stimulation. These cell-cycle promoters are also weakly repressed in positively selecting DP samples, suggesting that this break on the cell cycle might also occur during positive selection. However, we observe up-regulation of one G_1 cell-cycle promoter, cyclin D, in positive selecting thymocytes (Fig. 1, cluster 1a). Although the significance of cyclin D induction in DP thymocytes from TCR tg mice is not clear, it may be related to the observation that negative selection depends on activity of G_1 cell-cycle proteins (47, 48). As mentioned earlier, it is possible that a small proportion of thymocytes expressing positive selecting TCR transgenes undergo negative selection, and thus they may up-regulate G_1 cell-cycle proteins as part of their apoptosis program.

Positive Selection by MHC Class I Versus MHC Class II. Positive selection of $CD4^+CD8^+$ thymocytes on MHC class I and class II induces maturation toward the $CD8^+$ and $CD4^+$ T cell lineages, respectively. This observation raises the possibility that these selection signals might regulate distinct sets of genes. To investigate this possibility, we compared the expression profiles of DP thymocytes from class I-selecting (F5 and P14) and class II-selecting (5CC7 and AND) TCR tg mice (Figs. 5 and 6, which is published

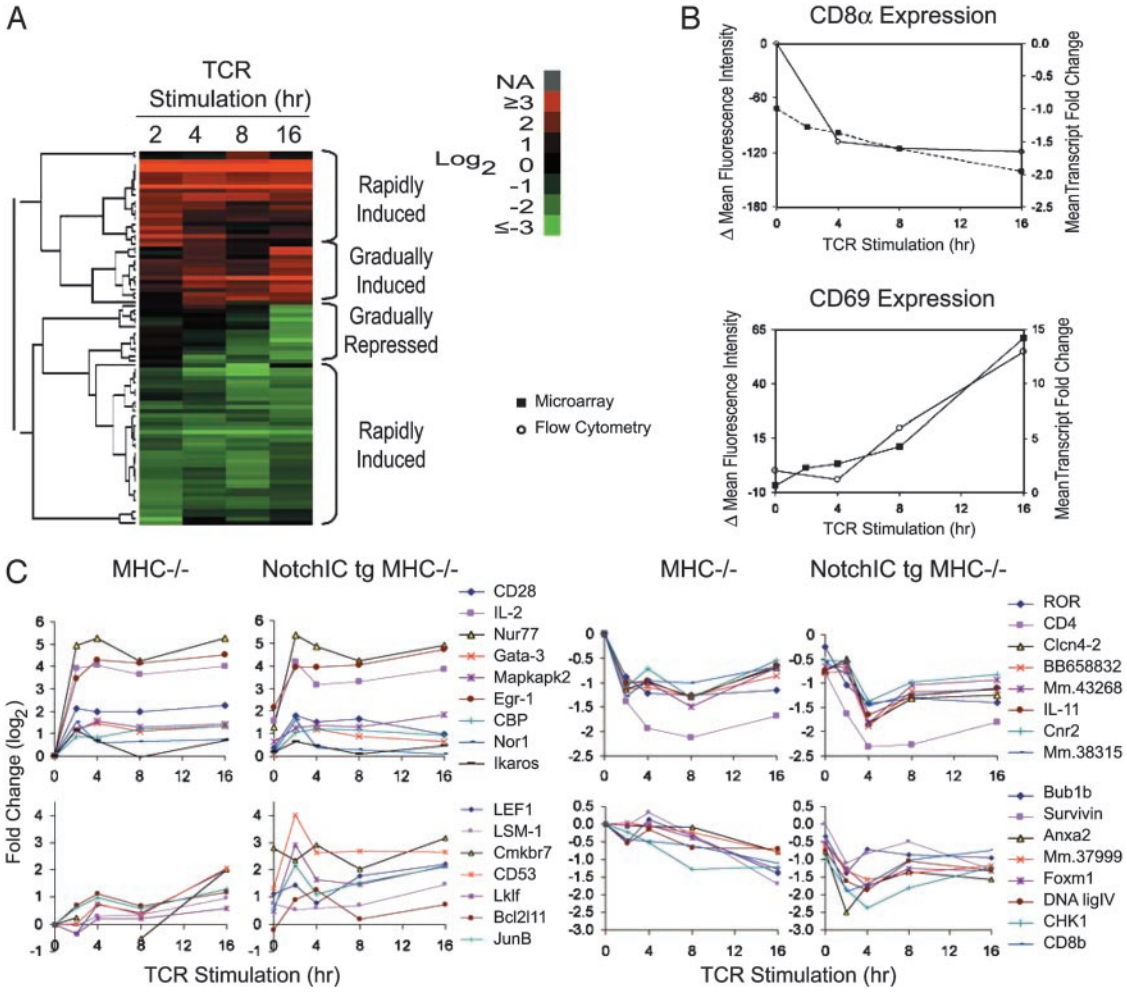

Fig. 2. Effect of Notch activity on TCR-driven gene expression changes. (*A*) Microarray analysis of MHC$^{-/-}$ thymocytes stimulated with plate-bound anti-TCR for 2, 4, 8, and 16 h. Ninety TCR-responsive genes were clustered as in Fig. 1. Four categories were apparent: rapidly induced or repressed and gradually induced (represented by two independent clusters) or repressed genes. (*B*) Microarray and flow cytometric analysis of CD8 and CD69 expression on TCR-stimulated MHC$^{-/-}$ thymocytes. (*C*) Effect of Notch activity on TCR-dependent rapidly and gradually modulated genes was determined by microarray comparison of normal littermate control MHC$^{-/-}$ and NotchIC tg MHC$^{-/-}$ thymocytes stimulated through TCR for 2, 4, 8, or 16 h. Graphs of representative genes of rapid (*Upper*) and gradual (*Lower*) inducers and repressors are displayed.

as supporting information on the PNAS web site). In general, DP thymocytes maturing toward either CD4$^+$ or CD8$^+$ single positive lineage had remarkably similar expression profiles (Fig. 5*a*, clusters 1 and 3). The lack of an obvious class I- or class II-specific gene expression profile suggests that the differences between positive selection on class I versus class II MHC may involve protein modifications and alterations in the activity of signaling pathways that are not reflected in global changes in gene expression.

It has been proposed that signals leading to CD4 development are stronger and/or of longer duration than signals leading to CD8 development (reviewed in refs. 49 and 50). If this hypothesis is correct, we might expect thymocytes being selected on class II MHC to induce and repress genes to a greater extent than thymocytes being selected on class I MHC. To compare the magnitude of gene expression changes in CD4$^+$CD8$^+$ thymocytes from class I- and class II-selecting TCR tg mice, we graphed the individual magnitudes of gene expression changes for each TCR tg strain against the average magnitudes of all four TCR tg strains and calculated the slope of the linear regression (Fig. 5*B*). Gene expression changes were greatest in 5CC7 (slope = 1.26), followed by AND and P14 (slope $\approx$ 1.0), and finally by F5 TCR tg thymocytes (slope = 0.78). Thus, the gene expression patterns are consistent with the quantitative model; however, analyses of additional TCR transgenic strains are required to clarify the relationships between signal strength, magnitude of gene expression changes, and lineage commitment.

Although the observed hierarchy of gene expression changes may reflect differences in the strength of selective signals received by individual TCR tg mice, it may also reflect heterogeneity in the thymocyte populations. For example, different TCR tg mice may differ with respect to the percentage of CD4$^+$CD8$^+$ cells responding to selection. To investigate this possibility, we analyzed the TCR tg thymocytes by flow cytometry for markers of TCR responsiveness. Both CD2 and CD5 were up-regulated in the entire CD4$^+$CD8$^+$ population in all of the TCR tg mice, indicating that the vast majority of thymocytes received a selective signal (Fig. 5*C*). We found CD5 levels on gated CD4$^+$CD8$^+$ thymocytes to be highest on 5CC7 and lowest on F5 TCR tg strains, further substantiating the trends we observed with microarray analysis (Fig. 5*B*). We also noted that the overall trend in CD2 protein as determined by flow cytometry and transcript expression levels as determined by DNA microarrays was very similar (Fig. 5*D*), providing additional confirmation of the reliability of our microarray analysis.

The Effect of Notch on TCR-Responsive Genes. The developmental fate regulator, Notch, has been reported to affect CD4 and CD8

development (3, 4, 10) and to down-modulate TCR signaling (7). To explore how Notch activity, TCR signaling, and positive selection are related, we examined the effect of Notch activity on TCR-induced transcription. To refine the analysis of TCR regulated gene expression changes for this purpose, we first resolved TCR-responsive genes into different kinetic categories by using cluster analysis. Four kinetic patterns, rapid induction, gradual induction, rapid repression, and gradual repression, were apparent (Fig. 2*A*). Induction of CD69 and down-regulation of CD4 and CD8 protein expression correlated well with gene expression changes measured by microarray analysis (Fig. 2*B* and Fig. 7, which is published as supporting information on the PNAS web site).

In parallel, we also examined TCR-induced gene expression changes in thymocytes expressing activated Notch. For this purpose, we crossed the activated Notch transgene onto a $MHC^{-/-}$ background. Like $MHC^{-/-}$ thymocytes, NotchIC tg $MHC^{-/-}$ thymocytes are arrested at the $CD4^{+}CD8^{+}$ stage because of lack of positive-selection signals (2). On TCR stimulation, NotchIC tg $MHC^{-/-}$ thymocytes in general responded as well or better to TCR stimulation than normal $MHC^{-/-}$ littermate control thymocytes (Fig. 2*C*). In particular, several gradually induced or repressed transcripts demonstrated more rapid kinetics and a greater magnitude of induction or repression in the presence of activated Notch (Fig. 2*C Lower*). In addition, several TCR-induced and -repressed transcripts, including nur77, egr-1, annexin-2, and CD4, were induced or repressed to some extent with NotchIC activity alone (Figs. 1 and 2*C*).

Notch activity does not affect the regulation of rapidly induced or repressed genes (Fig. 2*C Upper*); rather, the effect is specific for genes with more gradual kinetics. This finding suggests that Notch may sustain or enhance the effects of TCR signaling over time rather than directly promote TCR signaling. A related observation is that the gene expression changes induced by Notch overlap substantially with gene expression changes induced by positive selection (Fig. 1). Moreover, genes repressed by Notch correlated most closely with the genes most strongly repressed by positive selection. (Figs. 1 and 4, clusters 3a and 3b). Together these data support the notion that Notch signaling promotes thymocyte positive selection. Although some studies have suggested that Notch activity promotes CD8 development selectively (3, 9, 10), the lack of a class I- or class II-specific positive-selection gene cluster precludes any conclusion about lineage-specific promoting effects of Notch based on DNA microarray analysis.

Our observations are in contrast to those of Pear and colleagues (7), who suggest that Notch activity inhibits TCR signaling in thymocytes. This disparity may be the result of higher levels of Notch signal in the earlier study. Alternatively, the presence of MHC ligands and on-going positive selection may have affected the *in vitro* TCR responses in the earlier study.

By assessing gene expression in unstimulated NotchIC tg $MHC^{-/-}$ thymocytes, we found that Notch activity alone was sufficient to induce and repress a subset of genes, some of which were not significantly induced by TCR or positive-selection signals (Fig. 4, cluster 4). These included the well characterized Notch target HES-1 (51). In agreement with previous studies suggesting Notch autoregulation (4, 52, 53), we also observed the up-regulation of both endogenous Notch1 and Notch3 receptors. Notch activity also induced a notable number of cytokine-related genes. These gene products act at multiple levels of cytokine signaling and include the cytokine TGFα, the high-affinity IL-2Rα CD25, the downstream transcription factor IRF4, and the cytokine-signaling inhibitor SOCS-3. Thus, aside from its potential to modulate TCR signaling, Notch may also affect thymocyte development through cytokine signaling.

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