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In Vitro Transfection of Fresh Thymocytes and T Cells Shows Subset-Specific Expression of Viral Promoters

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We describe conditions under which exogenous DNA templates can be introduced for transient expression into primary murine T lymphocytes. T cells at various stages of development, including concanavalin A-activated splenic T cells, immature pre-T cells, and even small cortical thymocytes, could be successfully transfected. A variety of model DNA constructs were compared in which different viral promoter regions were used to drive expression of the chloramphenicol acetyltransferase (CAT) reporter gene. All showed enhanced expression in cells that had been acutely stimulated with the Ca^{2+} ionophore A23187 and phorbol ester as chemical proxies for T-cell receptor-mediated signals. In addition, splenocytes but not thymocytes required prior treatment with a mitogen and interleukin-2 in order to express these constructs, implying that even postmitotic thymocytes may be held in a quasiactivated state. A most striking result was the finding that the viral regulatory sequences in the Rous sarcoma virus long terminal repeat and the simian virus 40 early region were subject to sharply differential regulation, with a rank order that changed depending on the developmental stage of the T cells. The most immature thymic blasts and several lymphoma cell lines expressed the pRSV-Cat and pSV2-Cat constructs similarly, but cortical thymocytes exhibited a strong preference for pSV2-Cat. Splenic concanavalin A-stimulated blasts, on the other hand, slightly preferred pRSV-Cat, a tendency which became exaggerated in factor-dependent T-cell lines. The ratio of pRSV-Cat to pSV2-Cat expression varied according to cell type by as much as 500-fold. These results argue against a trivial linkage of promoter preference to cell cycle status but instead provide evidence that activation of T cells at distinct stages of differentiation results in the expression of different ensembles of nuclear regulatory proteins. In contrast to the simian virus 40 and Rous sarcoma virus promoter regions, the long terminal repeats of the retroviruses mink cell focus-forming virus and Akv were expressed well in all primary T-lineage cells. Thus, they represent excellent model promoters for engineering developmental stage-independent expression of exogenous genes in murine T cells.

Mature T lymphocytes comprise functionally distinct subsets with discrete roles in the regulation of the immune response. Although this specialization results from differences in gene expression, the signals which drive this differentiative process are only beginning to be understood. Transfection experiments with T-cell-specific genes have identified both *trans*-acting factors and *cis* regulatory regions which contribute to lineage-specific expression (reviewed in reference 59). In general, these experiments utilize immortalized cell lines which are more easily transfected than are primary T cells. However, although established cell lines are of tremendous value in the study of T-cell biology, it is likely that they differ in important ways from their normal counterparts. It is especially difficult to find appropriate models for cortical thymocytes, which are developmental intermediates at a critical three-way choice point in T-cell differentiation, poised between death on the one hand and maturation along one of at least two different lineages. Whereas most of these cells, in situ, are destined to die, the majority of cell lines established from them are rapidly dividing thymomas.

Alterations in the repertoire of transcription factors should both reflect and mediate the functional changes in developing T cells. Accordingly, there are striking differences in the abilities of various thymocyte subsets to express immune response-associated genes (5, 6, 47, 50, 51), which in some cases have been shown to correlate with the presence or absence of known transcription factors (11a, 45). Although the presence or absence of many transcription factors can be determined by DNA footprinting and electrophoretic mobility shift assays, such an experimental approach requires prior knowledge of the DNA region to be tested. Even when discrete protein-binding sites are demonstrated in a gene regulatory region, direct transfection experiments are often required to confirm that such sites are functionally important in vivo. In such cases, it would be advantageous to assay regulatory DNA sequences directly in the cells of interest rather than in model cell lines. This is absolutely necessary when it is the differences among constituents of the cell's transcriptional machinery in different developmental states that are being assessed. Therefore, we set out in this work to develop conditions for measuring transcriptional utilization of exogenous DNA templates in freshly isolated thymocytes and peripheral T cells.

As a first step, the transfectability of a variety of primary T-cell populations was assessed by measuring their ability to utilize two broadly expressed positive control promoters, the simian virus 40 (SV40) early promoter and the Rous sarcoma virus (RSV) long-terminal repeat (LTR), as well as the LTRs of two retroviruses implicated in thymic leukemia, mink cell focus-forming virus type 13 (MCF-13) and Akv. Our results demonstrate that thymocytes from both normal and SCID mutant mice express easily detectable levels of chloramphenicol acetyltransferase (CAT) activity following transfection under appropriate conditions. The MCF and

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Akv promoters are expressed well by all primary populations. On the other hand, the relative strength of the RSV and SV40 viral promoters is dependent on the type of T cell transfected. A strong preference for RSV over SV40 is seen in established T-cell lines which retain their interleukin-2 (IL-2) dependence, but both are expressed equally in cell lines which are factor independent for growth. By contrast, freshly isolated cortical thymocytes show a marked bias in favor of the SV40 early region over the RSV LTR. In general, after T cells migrate from the thymus, they lose their strong preference for the SV40 early promoter, a shift which probably reflects a developmental shift in the battery of *trans*-acting factors that they possess.

MATERIALS AND METHODS

Reagents. The phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and the calcium ionophore A23187 were used at final concentrations of 10 and 37 ng/ml, respectively. Concentrated stock solutions made up in dimethyl sulfoxide (DMSO) and stored at −20°C were diluted in growth medium immediately prior to use.

Cell lines. The IL-2-independent cell lines EL4.E1, Jurkat, BW5147, R1.1, AO1T-2.11 (53), 629, 705, and 728 (44) were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7), and antibiotics (full medium). The IL-2-dependent lines MTL2.8.2, CTLL-2, and HT-2 were maintained in full medium containing 5% conditioned medium (CM) from TPA-stimulated EL4.E1 cells as a source of IL-2. The IL-3-dependent premast cell line 32Dcl5 was grown in Dulbecco modified Eagle medium (DMEM) containing 10% FBS supplemented with 20% WEH1-3B CM as a source of IL-3.

All cell lines are of murine origin except Jurkat, which is a human cell line.

Plasmid DNA and transfections. Plasmids pSV2-Cat and pRSV-Cat were provided by B. Howard and C. Gorman (National Institutes of Health) and have been described previously (24, 25). pUC18-Cat contains a *Sph*I-*Bam*HI fragment from pSV2-Cat inserted into the *Sph*I-*Hin*dIII sites of pUC18. This construct contains only 22 bp of the SV40 enhancer region and therefore lacks effective enhancing activity (25). In plasmids pMCF-Cat and pAkv-Cat, the SV40 promoter has been removed from pUC18-Cat by digestion with *Pst*I and *Hin*dIII and replaced with *Pst*I-*Sma*I LTR fragments from the retroviruses MCF-13 and Akv mouse leukemia virus, respectively. Plasmids pMS1 and pAS1 contain 326-bp *Pst*I-*Dra*I enhancer fragments from MCF-13 and Akv, respectively, inserted into the *Xba*I site in the polylinker region of pUC18-Cat. They therefore include the MCF or Akv enhancer, with the SV40 promoter proper. The LTR fragments used in these constructs have been described in detail previously (29).

All plasmid DNAs were purified on cesium chloride-ethidium bromide density gradients before use.

Cell lines were transfected by DEAE-dextran facilitation essentially as described previously (42). Briefly, cells were washed once in full medium and then once in serum-free DMEM containing 10 mM HEPES, pH 7.0 (DMEM/H). After the second wash, cells were resuspended at 10^7/ml in DMEM/H containing 250 μg of DEAE-dextran (molecular weight, 2×10^6) per ml, 0.1 mM chloroquine, and 10 μg of supercoiled plasmid DNA per ml. Cells were put in a 37°C–7% CO_2 incubator for 60 min (30 min for EL4.E1 cells) with occasional swirling. The cells were then diluted with ~3 volumes of DMEM/H and pelleted. EL4.E1 cells were washed a further time in DMEM/H plus 150 U of heparin per ml to reduce cell clumping. Modifications of this protocol to allow efficient transfection of primary T cells and thymocytes are described below.

Twenty-four hours posttransfection, cells received either DMSO (solvent control) or TPA-A23187. Cells were harvested 18 h later, counted to determine viability, and lysed as previously described (42).

Calculation of relative promoter strength. Lysates from equal viable cell numbers were assayed for CAT activity in a 5-h assay as previously described (42). After autoradiography of the CAT assay thin-layer chromatography plate, spots corresponding to [^{14}C]chloramphenicol and its acetylated derivatives were cut out and counted. Background counts per minute from mock-transfected cells were subtracted from all experimental acetylated values, after which the percent acetylation was calculated. All samples with less than 0.01% acetylation were considered to be below the level of detection. CAT activity was then normalized to a constant number of viable cell equivalents, either 10^6 cells for lines or 10^7 cells for thymocytes and primary T cells. A major source of variability between experiments was the irreproducible survival of the cells through transfection and stimulation. CAT activity per viable cell tended to be higher in experiments in which the recovery of viable cells was lower. This effect was difficult to control but appeared to reflect subtle differences in the condition of the cells used in different experiments, not sample-to-sample variation within an experiment. The relative expression of different plasmids within an experiment was much more consistent. Thus, the relative promoter strength is presented as a ratio, R/S, which is the ratio of normalized CAT activity in cells transfected with pRSV-Cat to the activity in cells transfected with pSV2-Cat. All comparisons of promoter strength were made with cells that had been transfected on the same day with the different plasmids. At least two separate preparations of each plasmid were used with similar results.

Overall, the results reported were consistent over a period of 3 years and repeated in two different laboratories.

Thymocyte preparation. Thymuses were removed from young C57BL/6 (B6), BALB/c, and C.B-17 *scid/scid* (SCID) mice at 4 to 5 weeks of age, and single-cell preparations were made by pressing the thymuses through a fine wire mesh screen. Thymocytes were washed in full medium and were either transfected immediately or separated into subsets first. Because of the large numbers of cells required to detect expression of transfected DNA, low-yield methods like fluorescence-activated cell sorting could not be used. Size fractionation of thymocytes by centrifugal elutriation and complement elimination of $CD8^+$ cells were performed exactly as described previously (5). In most experiments, we found it convenient to separate large numbers of thymocytes into cortical and medullary populations by panning on peanut agglutinin (PNA)-coated petri dishes. PNA panning was done as described previously (10), with minor modifications. Petri dishes (150-mm diameter) were coated with PNA the previous day by incubation for 60 min at 25°C with 10 ml of a solution containing 20 μg of PNA per ml in 50 mM Tris–150 mM NaCl, pH 9.5. Plates were washed extensively with phosphate-buffered saline (PBS) containing Ca^{2+} and Mg^{2+} (PBS^+), overlaid with 10 ml of PBS^+–1% FBS, and stored at 4°C until use. For PNA fractionation, thymocytes were resuspended in PBS^+–5% FBS to a concentration of 2 ×

10^7/ml, and 10 ml was applied to each PNA-coated plate. The plates were incubated at 4°C for 60 min with occasional swirling. PNA^- cells were decanted, and the plates were washed twice with 10 ml of PBS^+–1% FBS. These two washes were combined with the PNA^- cells. To remove PNA^+ cells, the plates were incubated at 37°C for 10 min with 10 ml of PBS^+–0.2 M methyl-α-D-galactopyranoside. PNA^+ cells were then dislodged with a plastic cell scraper (Costar). The absence of azide in the PBS during panning results in strong binding of the PNA^+ cells to the plate, allowing vigorous washing of the plates. Thus, there is very little contamination of PNA^- cells in the PNA^+ fraction.

Fractionated thymocytes were washed several times with full medium and transfected as described above for cell lines, with the following modifications: (i) cells were transfected at a density of 10^8/ml (10^7/ml for SCID thymocytes), (ii) transfected thymocytes received TPA+A23187 2 to 5 h posttransfection, and (iii) stimulation with TPA+A23187 was for only 12 h before cells were harvested and assayed for CAT activity. The reduced time of incubation allowed even delicate thymocytes to be recovered with good viability. Murine cortical thymocytes could not be prestimulated with concanavalin A (ConA), as this treatment leads to rapid cell death (49a). The uptake and expression of plasmid DNA in thymocytes were similar whether or not the cells were cultured in the presence of 5 mM Zn^{2+} to inhibit endogenous nucleases (14a).

Spleen cell preparation. Spleens from 7- to 16-week-old mice were teased in full medium and pressed through a wire screen to produce a single-cell suspension. Erythrocytes were lysed in Tris-buffered ammonium chloride, and viable cells were further purified on a Ficoll-metrizoate cushion. B-cell-depleted spleen cells were prepared by two rounds of panning on goat anti-mouse immunoglobulin-coated plates. Spleen cells were transfected at a density of 10^8/ml for 25 min, after which they were washed and put into culture. Compared with these conditions, a cell density of 10^7/ml or incubation times greater than 30 min resulted in a 75% reduction of viable cells.

Twenty-four hours posttransfection, cells were stimulated with TPA+A23187. They were harvested 18 h later for CAT assay.

For experiments with ConA blasts, cells were cultured at 1×10^6 to 2×10^6/ml for 2 to 4 days in the presence of 4 μg of ConA (Vector) per ml. The cells were washed with PBS plus 25 μg of methyl-α-D-mannopyranoside per ml before transfection. Transfected cells were cultured in full medium plus 5% EL4 CM for 24 h before they were stimulated with phorbol ester and ionophore.

DNA extraction and quantitation. PNA^+ thymocytes and CTLL-2 cells transfected in parallel with pRSV-Cat or pSV2-Cat were analyzed 16 and 42 h later, respectively. One half of the cells were assayed for CAT activity. The other half were lysed by the method of Hirt (28a), and low-molecular-weight DNA was isolated. DNA from 3×10^5 cell equivalents was run in a 0.75% agarose gel and blotted onto a nylon filter (Nytran; Schleicher & Schuell). Plasmid sequences were detected by hybridization to a pSP65 derivative plasmid probe (pTN42) labeled with [α-^{32}P]dCTP by random priming. Hybridization and wash conditions were as described previously (41). After suitable autoradiographic exposures were obtained, the regions of the filter containing hybridized probe were excised and counted.

RESULTS

Cortical thymocytes can express exogenous DNA. The pivotal cell populations for analysis of the changes in gene regulation during T-cell development are thymocytes. These cells undergo dramatic changes in mitotic activity, responsiveness, and functional competence in the course of their differentiation and selection before leaving the thymus (reviewed in references 1, 19, 20, 49, 51, and 60). To establish model conditions for the study of immune response-associated gene regulation in thymocytes, we addressed the problems of viability, activation state, and functional heterogeneity in thymocytes.

First, cortical thymocytes, the cells at the point of choice between positive, negative, and failed selection, are particularly refractory to in vitro stimulation and particularly easy to kill by in vitro culture. To maintain their contribution to the results, we modified our standard transfection procedure to minimize the incubation time before assaying reporter gene expression. Control transfection experiments using the conditions described in Materials and Methods showed that when thymocytes were incubated with plasmid DNA in the presence of DEAE-dextran, they took up DNA in a time-dependent fashion and maintained an average of ~50 copies of plasmid DNA per cell for at least 24 h (data not shown). Second, since many function-associated genes are expressed in T cells only after activation, we compared CAT activity in transfected cells with and without a further activation step. Chemical proxies for T-cell receptor (TcR)-generated signals, namely, the calcium ionophore A23187 plus the phorbol ester TPA, were used to allow stimulation of immature and mature thymocytes alike (37, 51). Finally, because responses of total thymocyte populations are usually dominated by the minority of relatively mature thymocytes, we compared the transfectability of total thymocytes with that of thymocyte subsets fractionated by adherence to PNA. PNA^- cells are at least fivefold enriched for mature thymocytes, while PNA^+ cells are depleted of them. Usually over 90% of PNA^+ cells have the cortical $CD4^+$ $CD8^+$ (double-positive) surface phenotype, and all cells in this population are characteristically unable to respond to induction by expressing either IL-2 or the IL-2 receptor α chain (6, 37). By contrast, the PNA^- population is about fivefold enriched for cells that are competent in these inducible functions (6, 37, 51). This enrichment of functionally mature cells correlates well with corresponding four- to sixfold increases in the percentages of cells with $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ single-positive phenotypes. Figure 1A shows an example of the typical CD4/CD8 staining profiles of the PNA-fractionated populations used for transfection. The results of transfecting these thymocytes, and a summary of four such experiments, are presented in Fig. 1B and 2 and in Table 1.

Thymocytes were clearly able to express CAT from exogenous DNA templates, particularly after a 12-h stimulation with TPA+-A23187. The cells responsible for this expression appeared, surprisingly, to be cortical thymocytes. PNA^+ thymocytes expressed 2 to 20 times more CAT activity per cell than did total thymocytes or PNA^- cells, depending on the construct used (Fig. 1B; compare SV2 lanes; Tables 1 and 2). This is unlikely to reflect the presence of small numbers of contaminating $CD4^+$ $CD8^-$ cells in the PNA^+ fraction, since the ability to express CAT was negatively correlated with the presence of such mature-type cells. $CD4^+$ $CD8^-$ cells in general are at least fivefold more concentrated in the PNA^- fraction, which is less able to express CAT. Indeed, as our fractionation procedure leaves

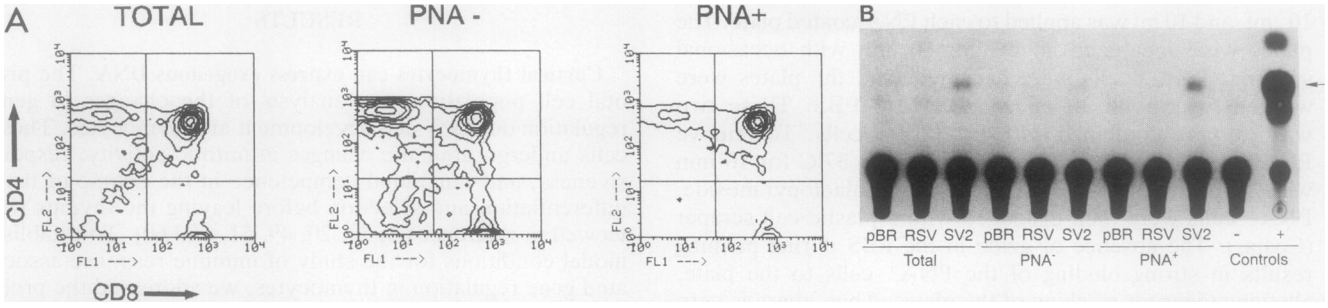

FIG. 1. Assay of CAT activity in thymocytes transfected with pRSV-Cat and pSV2-Cat. (A) CD4/CD8 staining profiles of starting populations. Total thymocytes, PNA^- cells, and PNA^+ cells from 4-week-old B6 mice were stained simultaneously with phycoerythrin-conjugated anti-CD4 and fluorescein isothiocyanate-conjugated anti-CD8 monoclonal antibodies (Becton Dickinson) and analyzed on a FACScan (Becton Dickinson). The distribution of cells is representative of four experiments. The fraction of cells in each population is as follows: total, 13% $CD4^+$, 81% $CD4^+CD8^+$, 2% $CD4^-CD8^-$, 4% $CD8^+$; PNA^-, 37% $CD4^+$, 47% $CD4^+CD8^+$, 4% $CD4^-CD8^-$, 12% $CD8^+$; PNA^+, 8% $CD4^+$, 90% $CD4^+CD8^+$, 0.4% $CD4^-CD8^-$, 2% $CD8^+$. (B) CAT assay of transfected thymocytes after 12 h of stimulation with phorbol ester and ionophore. Total PNA^- and PNA^+ thymocytes whose staining profiles are shown in panel A were transfected with pBR322 (pBR), pRSV-Cat (RSV), or pSV2-Cat (SV2) by DEAE-dextran facilitation. Cells received TPA and A23187 2.5 h posttransfection; they were harvested after 12 h of stimulation, and lysates from 3×10^7 cells were tested for CAT activity in a 5-h assay. This is experiment 1 in Table 1. Lanes marked − and + are negative and positive assay controls, respectively. The arrow indicates the primary reaction product.

a much larger percentage of $CD4^+$ $CD8^+$ (PNA^+-type) contaminants in the PNA^- pool (Fig. 1A; see Materials and Methods), it is possible that cortical-type cells even account for some of the transfectability in the mature cell population. This result contrasts dramatically with the behavior of PNA^+ and PNA^- fractionated cells in typical assays of immune function, in which PNA^+ cells are almost completely devoid of activity and the enrichment of responsiveness in the PNA^- fraction is conspicuous (6, 37). Thus, the fractionation behavior of the transfectable thymocytes is opposite to that of functionally competent cells.

The ratio of pRSV-Cat activity to pSV2-Cat activity in the transfected thymocytes provided evidence that most of the thymocytes expressing exogenous DNA were physiologically distinct from the T-lineage cell lines that have been transfected before. Unlike the factor-dependent cell line MTL.2.8.2 or the EL4.E1 thymoma (41; see below), these cells showed a strong preference for the SV40 early promoter over the RSV LTR (Fig. 1B). The ratio of pRSV-Cat activity to pSV2-Cat activity (R/S ratio) was ≤0.05 for both PNA^+ thymocytes and the total thymocyte population whose response they dominated (Table 1). These results did not reflect differential uptake or retention of the two plasmids by thymocytes, as we shall show below (see Fig. 6).

Notably, the mature thymocytes showed a less marked preference for pSV2-Cat in cases in which their transient expression could be monitored at all. PNA^- fractions typically contained too many cortical cells to give clear results. However, cortical thymocytes could be removed completely by cytolytic elimination with anti-CD8 plus complement. The resulting population of mature $CD4^+$ $CD8^-$ cells and $CD4^-$ $CD8^-$ cells was then able to express pRSV-Cat one-third as well as pSV2-Cat. The distinctive, much stronger bias for pSV2-Cat over pRSV-Cat in the cortical thymocyte population provides further evidence that the transfect-

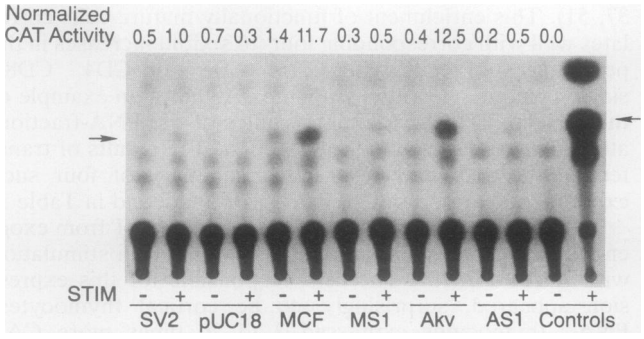

FIG. 2. Evidence that endogenous murine retrovirus LTRs are stronger promoters than the SV40 early promoter in cortical-type thymocytes. PNA^+ thymocytes from 5-week-old B6 mice were transfected with various promoter-CAT constructs as indicated. Three hours posttransfection, cells received DMSO (−) or TPA plus A23187 (+). Twelve hours later, they were harvested for CAT assay. CAT activity is presented as normalized units per 10^7 cell equivalents, where 1 U is the activity in the stimulated pSV2-Cat sample, calculated after subtracting the background radioactivity of the negative control. Arrows indicate the primary reaction product.

TABLE 1. Preference of transfected thymocytes for the SV40 early promoter[a]

Expt	Strain	Cells	CAT activity[b]		
			RSV	SV40	R/S[c]
1	B6	Unfractionated	<0.005	0.043	<0.12
		PNA^+	<0.006	0.080	<0.075
		PNA^-	<0.005	0.015	<0.33
2	B6	PNA^+	0.051	1.1	0.05
		PNA^-	0.025	0.32	0.08
3	BALB/c	PNA^+	<0.008	0.32	<0.025
		PNA^-	<0.005	0.022	<0.23
4	B6	PNA^+	0.032	2.2	0.015
		PNA^-	ND	0.1	
5	B6	$CD8^-$[d]	0.016	0.049	0.33

[a] Thymocytes, either unfractionated, PNA panned, or antibody-plus-complement eliminated, were prepared from the indicated strains of mice as described in Materials and Methods. Each population was then transfected with pRSV-Cat and pSV2-Cat. Two hours posttransfection, cells received TPA plus A23187. Cells were harvested 12 h later, and lysates from equal cell numbers were assayed for CAT.

[b] Expressed as percent acetylation per 10^7 viable cell equivalents in a 5-h assay. ND, not done.

[c] R/S = (CAT activity with pRSV-Cat)/(CAT activity with pSV2-Cat).

[d] These cells were 58% $CD4^+$, 41% $CD4^-CD8^-$, <1% $CD8^+$ when transfected.

TABLE 2. Evidence that cells with a strong preference for the SV40 regulatory region are small cortical thymocytes[a]

Elutriated fraction	Cell	CAT activity[b]			% of recovered cells[c] (iv)	Calculated % of CAT activity in thymus[d]	
		RSV (i)	SV40 (ii)	R/S[e] (iii)		RSV (v)	SV40 (vi)
M/L	PNA$^+$	0.40	1.24	0.32	5.3	60.3	27.6
M/L	PNA$^-$	0.15	0.33	0.45	3.3	14.4	4.6
S/I	PNA$^+$	0.009	0.19	0.05	81.3	21.0	64.4
S/I	PNA$^-$	0.015	0.08	0.19	10.1	4.3	3.4

[a] Thymocytes were separated into two size fractions by centrifugal elutriation. In this experiment, 8.6% of the cells were recovered in the M/L fraction. Cells were further fractionated by PNA panning before being transfected with pRSV-Cat and pSV2-Cat.

[b] Expressed as percent acetylation per 10^7 viable cell equivalents in a 5-h assay. All cells were stimulated with TPA plus A23187 for 12 h prior to assay.

[c] The recovery of viable cells from elutriation was 98% of input. The recovery from the PNA panning was 41% from the M/L fraction and 49% from S/I fraction. Yield calculations in the last three columns are presented on the assumption that losses are random.

[d] Calculated for each fraction (n) as

$$100 \times \frac{(\text{CAT activity})_n \times (\%\text{ of recovered cells})_n}{\sum_{m=1}^{4} [(\text{CAT activity})_m \times (\%\text{ of recovered cells})_m]}$$

where m is the number given to each of the four fractions.

[e] R/S = (CAT activity with pRSV-Cat)/(CAT activity with pSV2-Cat).

able cells enriched therein are not contaminants from another thymocyte subset.

The expression of pSV2-Cat in cortical thymocytes was surprising given the developmental end stage that most of these cells represent. To rule out the possibility that this expression was an artifact due to aberrant transcriptional initiation from plasmid sequences, PNA$^+$ cells were transfected with an enhancerless derivative of this plasmid. pUC18-Cat contains only the proximal 22 bp of the SV40 72-bp repeats and lacks a functional enhancer (25). When transfected into PNA$^+$ thymocytes, this deletion results in a sixfold decrease in expression relative to expression from the intact SV40 early promoter (Fig. 2; compare SV2 lanes with pUC18 lanes; also data not shown). Therefore, utilization of the SV40 early promoter in these cells is dependent on a functional enhancer.

To explore further whether the transfectable cells in the PNA$^+$ population were typical cortical thymocytes, we subjected them to additional fractionation steps. The relatively low levels of CAT activity expressed per cell made it necessary to transfect considerable numbers of thymocytes to achieve results above the threshold of detection. Multiparameter fluorescence-activated cell sorting would be completely inadequate to supply these cell numbers. However, a biologically significant difference among cortical thymocytes is correlated with cell size. Although most cortical thymocytes are small, postmitotic, and apparently terminal cells, some are large blast cells with the ability to expand and generate double-positive and single-positive populations upon transfer into a normal thymus (26, 39). To take advantage of this difference, we combined PNA panning with size fractionation by centrifugal elutriation (10, 32, 36, 37). We have previously shown that approximately 90% of thymocytes belong to the small and intermediate (S/I) classes, and almost all of them are in G_0 (5). On a per-cell basis, they contain 10-fold less cytoplasmic RNA than do medium and large (M/L) thymocytes. These M/L cells are mostly in the G_1, S, and G_2+M phases of the cycle and contain more than two-thirds of the RNA found in the thymus even though they represent only ~10% of total thymocytes (32).

To test whether these large cycling cells were responsible for the expression of transfected plasmid DNA, we PNA fractionated thymocytes which had first been separated into S/I and M/L fractions by centrifugal elutriation. The resulting four populations were transfected with either pRSV-Cat or pSV2-Cat and then stimulated for 12 h with TPA and A23187 before being assayed. The results (Table 2) show that both large and small cells contribute to the transient expression pattern but that the cells in the two size classes behave differently. As predicted, the blast cells expressed more CAT activity per cell than did the small cells (Table 2, columns i and ii). However, the blast cells showed only a slight bias in favor of SV40 expression over RSV, with R/S values of 0.3 to 0.5 (Table 2, column iii). Thus, they could not be responsible for the predominance of SV40 expression in the total thymocyte or total PNA$^+$ cell populations (cf. Fig. 1B). The cells with the most marked preference for SV40 over RSV were in fact found in the S/I PNA$^+$ fraction (R/S of 0.05). Although these small cells were less efficient per cell than the blasts in transient expression, in the aggregate they could account for almost two-thirds of the SV40 early region utilization by thymocytes overall (Table 2, column vi). Thus, cells with the size and surface properties of small cortical thymocytes are competent to express exogenous DNA and account for the distinctive preference of bulk thymocytes for the SV40 over the RSV promoter.

Murine retrovirus LTRs are extremely strong promoters in thymocytes. Although both the RSV LTR and the SV40 early promoter can be utilized by freshly isolated T cells, neither virus normally infects mice, and thus these promoters are not operating in an evolutionarily selected cellular context. By comparison, LTRs from murine leukemia viruses would be expected to function even better than these promoters in transfected thymocytes and T cells. Figure 2 shows a representative CAT assay of PNA$^+$ thymocytes that had been transfected with constructs containing promoters from two murine thymic leukemia viruses. The plasmid pMCF-Cat contains the intact LTR, which provides both a promoter and an enhancer, from the lymphomagenic murine leukemia virus MCF-13 (61). Recombinant MCF retroviruses of this type cause primarily cortical thymomas in the AKR strain of mice (11, 13, 43). pAkv-Cat contains the LTR from a nonpathogenic, endogenous AKR retrovirus, Akv. When introduced into PNA$^+$ thymocytes, both of these plasmids were expressed at high levels, yielding even more CAT activity than did the intact SV40 early promoter (Fig. 2;

compare SV2, MCF, and Akv lanes). This increase in expression depends on both the enhancer and promoter regions of the viral regulatory sequence, because hybrid constructs containing the SV40 proximal promoter and either the MCF-13 or the Akv enhancer region (pMS1 and pAS1, respectively) were expressed no better than was pSV2-Cat (Fig. 2). Examples of such promoter-dependent enhancer activity have been reported previously (3).

In each case, expression of the viral promoter was significantly enhanced if the cells were induced with TPA plus A23187 for 12 h after the transfection (compare + and − lanes in Fig. 2). This finding suggests that rate-limiting transcription factors for all of these viral regulatory sequences may be among the set that can be activated in T cells in response to elevated Ca^{2+} concentrations and activation of protein kinase C. Furthermore, if the transfectable cells in the PNA^+ population are representative of cortical thymocytes generally, it suggests that these cells are able to display some inducible changes in transcription factor expression even if these are not sufficient to activate transcription of the IL-2 and IL-2 receptor α-chain loci (6, 51).

Immature thymocytes show little preference for RSV or SV40. Small cortical thymocytes are the product of a complex series of proliferative and differentiative processes that act on thymocytes over a period of at least a week (55). Their properties may not, therefore, be truly those of immature cells but rather those of cells at a penultimate choice point. Our results with enriched thymic blast cells, which include the immediate precursors of small cortical cells, suggested that the strongly biased R/S ratio might not be characteristic of earlier precursors (Table 2). To examine this question more directly, we tested the ability of immature, TcR-negative $CD4^-$ $CD8^-$ (double-negative) thymocytes to express our panel of viral promoters. These cells are normally a small minority of thymocytes which must be isolated via prolonged incubation with antibodies and complement. To avoid potential artifacts in preparing immature cells, we used thymocytes from C.B-17 *scid/scid* mice. Mice homozygous for the *scid* mutation (SCID) are defective in their ability to rearrange functional TcR and immunoglobulin genes, and the only viable cells in their thymuses are those that have not progressed beyond the $CD4^-$ $CD8^-$ stage of development (4). As shown in Fig. 3A, SCID thymocytes were at least as transfectable as normal thymocytes overall when assayed with the strong pMCF-Cat construct. Unlike most normal thymocytes, however, SCID thymocytes were capable of expressing the pRSV-Cat and pSV2-Cat constructs about equally well, albeit both less efficiently than the MCF construct (Fig. 3B and data not shown; R/S ratios of 1.3 and 0.8 in two experiments). Thus, the immature thymocytes present in the SCID mouse, like the dividing blast cells in the normal thymus, can be transfected for transient expression but exhibit an R/S ratio that is clearly distinct from that of the dominant transfectable population of normal cortical thymocytes.

Transfected spleen T cells differ from thymocytes by their requirement for prior activation and their slight preference for the RSV LTR. Because thymocytes are intermediates in T-cell development, it was of interest to determine whether their mature progeny exhibited the same strong bias in favor of the SV40 early promoter. Preliminary experiments used B-cell-depleted spleen cells (65 to 85% $Thy1^+$) or unfractionated splenocytes as DNA recipients. Cells were transfected with CAT constructs, cultured for 24 h, and then stimulated overnight with TPA and A23187 before being assayed. In contrast to freshly isolated thymocytes, no CAT activity

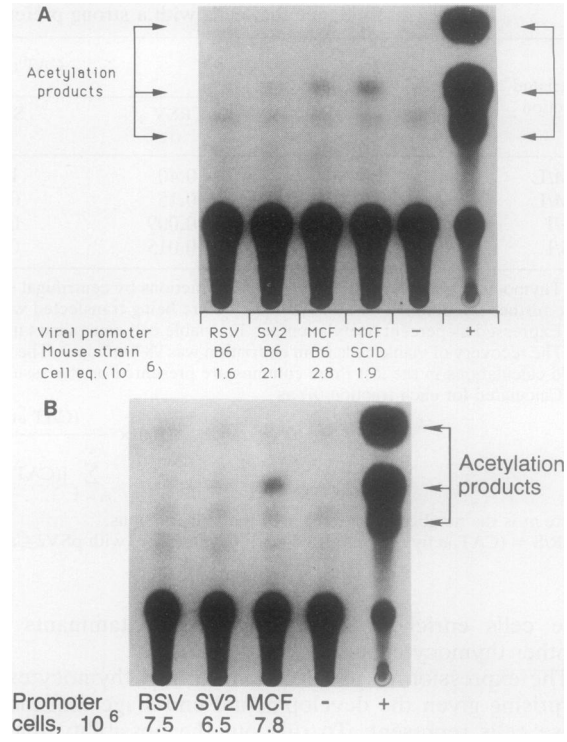

FIG. 3. CAT expression in transfected SCID thymocytes. (A) SCID thymocytes express transfected DNA similarly to normal thymocytes. Unfractionated thymocytes from 4- to 6-week-old B6 and SCID mice were transfected with the indicated plasmids, and 4 h later they were stimulated with TPA plus A23187. Cells were harvested 12 h later, and extracts from 1.6×10^6 to 2.8×10^6 cells, as indicated, were assayed for CAT activity. (B) Expression of different viral promoters in SCID thymocytes. Thymocytes from 3- to 4-week-old SCID mice were transfected with the indicated plasmids, and 4 h later they were stimulated with TPA plus A23187. Cells were harvested after 12 h of stimulation, and extracts from the indicated number of cells were assayed for CAT activity. A longer exposure (not shown) demonstrated that SV2 was expressed slightly better than RSV in this experiment, although in at least one other experiment, RSV was expressed slightly better than SV2. Lanes marked − and + are negative and positive assay controls.

could be detected in the lysates of these cells (Table 3, experiments 1 and 2, and data not shown). Control experiments ruled out the possibility that these cells were deficient in DNA uptake. Gel blot analysis of total nuclear DNA isolated 2 days posttransfection showed that cells contained, on average, 5 to 10 copies of plasmid DNA (not shown), which is 5- to 10-fold less than found in thymocytes. Freshly isolated spleen cells, however, have a low cytoplasmic-to-nuclear ratio, which might limit DNA uptake to a level below that required for detectable expression of the CAT reporter gene. To circumvent this problem and to increase the proportion of T cells in the population, B-cell-depleted splenocytes were first cultured for 3 days in the presence of ConA. These mitogen-activated blasts (>95% $Thy1^+$) were then transfected with CAT plasmids and cultured for another 24 h before being treated with phorbol ester and ionophore. Cells were harvested for assay after 18 h of stimulation. As shown in Table 3 and Fig. 4, this protocol resulted in easily detectable CAT expression by spleen T cells. Like thymocytes, these cells expressed the MCF and Akv LTR constructs very well, about sixfold better than either of the other

TABLE 3. Preference of transfected spleen ConA blasts for the RSV LTR

Expt	Spleen cells[a]	Treatment[b]	CAT activity[c]		
			RSV	SV40	R/S[d]
1	B cell depleted	None	0	0	
2	B cell depleted	None	0	0	
	B cell depleted	3d ConA[e]	0.40	0.043	9.3
3	B cell, CD4 depleted	3d ConA	20.0	9.2	2.2
	B cell, CD8 depleted	3d ConA	3.7	2.2	1.7
4	Whole spleen	3d ConA[f]	0.83	0.85	1.0

[a] Spleen cells from B6 mice were depleted of B cells by panning on anti-immunoglobulin-coated plates except in experiment 4. In experiment 3, these cells were also depleted of either $CD4^+$ or $CD8^+$ cells by antibody-plus-complement elimination.

[b] Purified cell populations were transfected with pRSV-Cat and pSV2-Cat either immediately after isolation or after 3 days of culture with 4 μg of ConA per ml (3d ConA). Twenty-four hours posttransfection, cells received TPA plus A23187; they were harvested for assay 18 h later.

[c] Expressed as percent acetylation per 10^7 viable cell equivalents in a 5-h assay.

[d] R/S = (CAT activity with pRSV-Cat)/(CAT activity with pSV2-Cat).

[e] These cells were 88% $CD8^+$, 9% $CD4^+$ at the time of transfection.

[f] These cells were 47% $CD8^+$, 30% $CD4^+$ at the time of transfection.

constructs. However, in contrast to cortical thymocytes, these cells used the RSV LTR at least as efficiently as the SV40 early region, with R/S ratios ranging from 1.0 to a high value of 9.3. While these cells were robust enough to allow both a longer recovery time after transfection and a longer period of stimulation than did thymocytes, the altered conditions were not the cause of their different pattern of promoter utilization. When day 3 ConA blasts were transfected and stimulated exactly like thymocytes, they maintained R/S values near 1, even though the absolute levels of expression were somewhat lower (data not shown). Stimulation with ConA plus IL-2 led to the outgrowth of $CD8^+$ T cells primarily, and in a direct comparison, the modest preference for the RSV LTR was somewhat more pronounced in $CD8^+$ cells than in $CD4^+$ cells (Table 3, experiment 3). Nevertheless, strong expression of pRSV-Cat at least equal to that of pSV2-Cat remained a general feature of both subsets of ConA blasts.

All of the data presented in Table 3 are from ConA blasts further stimulated with TPA and A23187. As shown in Fig. 4

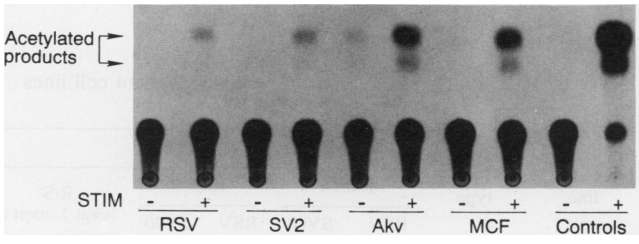

FIG. 4. Murine retroviral LTRs function efficiently in spleen ConA blasts. Spleen cells from 7-week-old B6 mice were cultured for 3 days in the presence of ConA. Activated cells were then transfected with the indicated plasmids. Twenty-four hours after transfection, cells were either stimulated with TPA plus A23187 (+) or left unstimulated (−). The cells were harvested 18 h later, and ~6 × 10^6 cells were assayed for each sample. The absolute percent acetylation values for the samples were as follows: RSV unstimulated, <0.01; RSV stimulated, 0.5; SV2 unstimulated, <0.01; SV2 stimulated, 0.47; Akv unstimulated, 0.13; Akv stimulated, 3.0; MCF unstimulated, 0.05; MCF stimulated, 2.9.

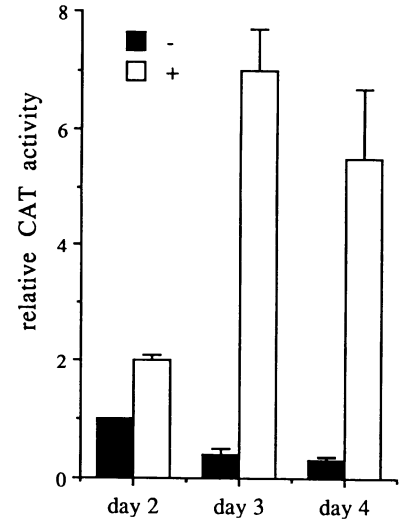

FIG. 5. Effect of ConA preculture on spleen T-cell CAT expression. B-cell-depleted spleen cells were cultured in the presence of ConA for 2, 3, or 4 days. On the day indicated, cells were harvested and transfected with pRSV-Cat. Transfected cells were cultured for an additional 24 h in the presence of IL-2 before receiving DMSO (−) or TPA and A23187 (+). After 18 h of stimulation, equal numbers of cell equivalents were assayed for CAT activity. Thus, day 2 ConA blasts were actually assayed 4 days after removal from the mouse. Relative CAT activity was calculated by normalizing the actual percent acetylation of each sample with respect to the value for the day 2 (−) sample, which was arbitrarily set to 1.0. Data are presented as means and ranges of two independent experiments.

and 5, without this treatment blasts produced detectable, but substantially lower, levels of CAT activity. Thus, even in the physiological context of the proliferating ConA blast, all four viral promoters behaved as elements acutely dependent on A23187-TPA induction. We have examined two other variables which affect the detection of reporter gene expression in spleen T cells. The duration of preculture in ConA prior to transfection strongly affected the level of CAT activity. Figure 5 shows that transfectability of the cells, as measured later by inducible CAT expression, increased sharply between 2 and 3 days of preculture in ConA and remained high for at least another 24 h. Inclusion of IL-2 in the culture medium after transfection resulted in the recovery of 5 to 10 times more CAT activity in day 3 and day 4 ConA blasts but had little effect on day 2 ConA blasts (data not shown). Thus, the 3-day preculture with ConA seemed to be required to confer the competence to express exogenous DNA on the splenic T cells, in a process distinct from the further modulation of expression level via A23187-TPA induction or via IL-2-dependent growth.

Growth factor-dependent T-cell lines exhibit a strong preference for the RSV LTR. Established clonal cell lines, continuously growing in response to IL-2, were examined to compare their behavior with that of freshly isolated T cells. Transfection of several nontransformed, IL-2-dependent cell lines with pRSV-Cat and pSV2-Cat revealed a marked preference for the RSV LTR in these cells (Fig. 6 and 7; Table 4). R/S values ranged from 11 to 50, irrespective of whether or not the cells were stimulated with phorbol ester and ionophore (Fig. 6 and data not shown). The cell lines tested included a pair of cytotoxic T-lymphocyte lines, CTLL-2 and MTL2.8.2, and an IL-4-inducible Th2-like line,

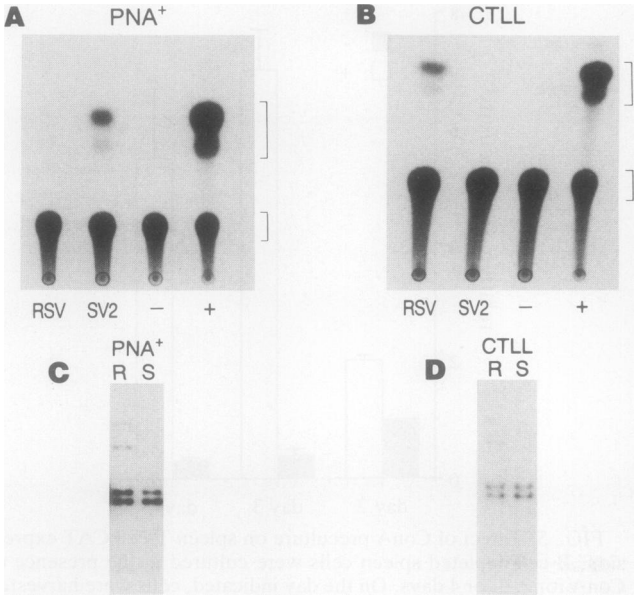

FIG. 6. Evidence that the disparate promoter preferences of transfected PNA^+ thymocytes and CTLL-2 cells are not due to differences in plasmid copy number. BALB/c PNA^+ thymocytes and CTLL-2 cells were transfected in parallel with pRSV-Cat and pSV2-Cat. Cells were stimulated as described in Materials and Methods and harvested after either 16 h (thymocytes) or 42 h (CTLL-2 cells) of culture. One half of the cells (1.1×10^7 thymocytes and 1.7×10^6 CTLL-2 cells) were assayed for CAT activity. Low-molecular-weight plasmid DNA was extracted from the other half of the cells by the method of Hirt (28a). DNA from 3×10^5 cell equivalents was electrophoresed, transferred to a nylon filter, and hybridized with a probe for the pBR322-derived sequences as described in Materials and Methods. (A and B) CAT assays from extracts of transfected PNA^+ thymocytes (A) and CTLL-2 cells (B). The thymocyte and CTLL-2 extracts were assayed for CAT activity under the same conditions but analyzed on separate chromatography plates. – and + indicate the negative and positive assay controls, respectively. The upper bracket indicates the major reaction products, and the lower bracket indicates the unreacted [^{14}C]chloramphenicol. (C and D) DNA samples extracted from cells assayed in panels A and B: (C) PNA^+ thymocytes; (D) CTLL-2 cells. The samples shown in panels C and D were analyzed on the same gel and hybridized simultaneously, but panel C is from an autoradiographic exposure time of 48 h, whereas panel D is from an autoradiographic exposure of 2 h. Lanes: R, DNA from cells transfected with pRSV-Cat; S, DNA from cells transfected with pSV2-Cat.

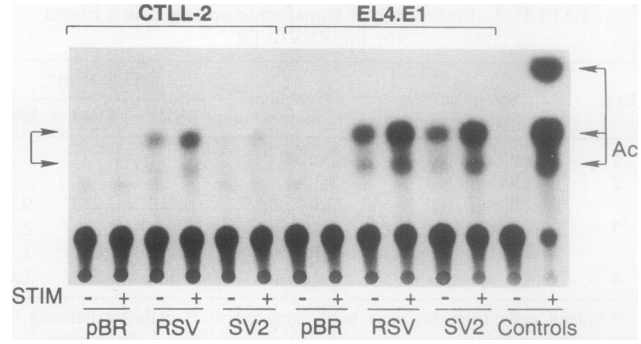

FIG. 7. Comparison of promoter preference in the CTLL-2 and EL4.E1 cell lines. The IL-2-dependent line CTLL-2 and the IL-2-independent thymoma EL4.E1 were transfected with pBR322, pRSV-Cat, or pSV2-Cat as indicated. Twenty-four hours posttransfection, one half of the cells were stimulated with TPA plus A23187 (+) and the remaining half received DMSO as a solvent control (–). Cells were harvested for assay 18 h later.

HT-2, suggesting that the preference for RSV over SV40 is not restricted to a particular T-cell sublineage.

Similar results were also obtained with the IL-3-dependent premast cell line 32Dcl5. Although this line is presumably of a myeloid lineage, it can be induced to express IL-4 like a Th2 cell (7, 42). Lines similar to this have been shown to express low-affinity IL-2 receptors and may gradually be adapted to growth in IL-2 (33, 35). Like the IL-2-dependent lines, 32Dc15 cells are absolutely dependent on the continued presence of an appropriate growth factor, and they die rather than arrest their growth when IL-3 is depleted. When these cells were transfected with pRSV-Cat and pSV2-Cat, they also exhibited a 20-fold preference for the RSV LTR (Table 4).

The sharply contrasting R/S ratios of cortical thymocytes and factor-dependent cells were not due to differential DNA uptake. This is illustrated by the experiment shown in Fig. 6. PNA^+ thymocytes and CTLL-2 cells differ greatly from one another in their overall efficiencies in DNA uptake (compare Fig. 6C [48-h exposure] with Fig. 6D [2-h exposure]), but in each cell type the levels of pRSV-Cat DNA that were taken up and retained were indistinguishable from the levels of pSV2-Cat DNA. An overwhelming preference for pSV2-Cat expression was shown by the thymocytes (Fig. 6A), and an overwhelming preference for pRSV-Cat expression was shown by the CTLL-2 cells (Fig. 6B), at the same times of incubation when they demonstrably contained equal amounts of pSV2-Cat and pRSV-Cat DNA. The characteristic R/S ratios of the different cell types must therefore reflect differential promoter utilization.

Similar results demonstrating a preference for the RSV LTR over the SV40 early promoter have been reported by Gorman and coworkers for a variety of primate and rodent fibroblast lines, employing both transient and stable expression systems (23, 24). However, as discussed below, for murine T-cell lines, such a strong preference for the RSV LTR may be restricted to a discrete activation state associated with factor-dependent growth.

IL-2-independent T-cell lines show little promoter preference. Malignant leukemia and lymphoma cell lines of T-lineage origin are frequently used in transfection studies to

TABLE 4. Preference of growth factor-dependent cell lines for the RSV LTR[a]

Cell line	Cell type	CAT activity[b]				R/S[c] (expt 1, expt 2)
		Expt 1		Expt 2		
		RSV	SV40	RSV	SV40	
CTLL-2	CTL[d]	11.0	1.0	2.0	0.18	11, 11
MTL2.8.2	CTL	1.1	0.034	0.32	<0.01	32, >32
HT-2	Th2	0.14	0.0025	3.3	0.1	56, 33
32Dc15	Premast	20	0.81	9.5	0.43	25, 22

[a] Exponentially growing cells were transfected with pRSV-Cat or pSV2-Cat, and 24 h later cells received TPA plus A23187. Cells were assayed for CAT 18 h later.

[b] Expressed as percent acetylation per 10^6 viable cell equivalents in a 5-h assay.

[c] R/S = (CAT activity with pRSV-CAT)/(CAT activity with pSV2-Cat).

[d] CTL, cytotoxic T lymphocytes.

TABLE 5. Evidence that growth factor-independent cell lines show little promoter preference when transfected with pRSV-Cat and pSV2-Cat

Cell line	Type	Stimulation[a]	CAT activity[b]				R/S[c] (expt 1, expt 2)
			Expt 1		Expt 2		
			RSV	SV40	RSV	SV40	
EL4.E1	Thymoma	−	0.87	0.21	4.0	1.6	4.1, 2.4
		+	2.4	0.80	42.0	30.0	3.0, 1.4
Jurkat	T-cell leukemia	−	5.8	2.0	0.73	0.15	2.9, 4.9
		+	9.3	12.5	2.0	2.3	0.7, 0.9
BW5147	Thymoma	−	0.36	0.24	0.70	0.30	1.5, 2.3
		+	2.6	6.3	4.2	4.0	0.4, 1.0
AO1T-2.11	T hybridoma	−	0.55	0.22	0.55	0.22	2.5, 2.5
		+	1.0	0.40	1.1	0.65	2.5, 1.7
728	Thymoma	−	0.08	0.08	0.025	0.045	1.0, 0.6
		+	0.26	0.34	0.13	0.09	0.8, 1.4
629	Thymoma	−	0.30	0.46	0.03	0.09	0.6, 0.3
		+	1.2	0.9	0.15	0.05	1.3, 3.0
R1.1	Immature thymoma	−	0.01	0.002	0.006	0.001	5.0, 6.0
		+	0.008	0.002	0.02	0.009	4.0, 2.2
705	Immature thymoma	−	0.006	0.009	0.002	0.009	0.7, 0.2
		+	0.034	0.014	0.008	0.007	2.4, 1.1

[a] −, unstimulated; +, 18-h stimulation with TPA plus A23187.
[b] Expressed as percent acetylation per 10^6 viable cell equivalents in a 5-h assay.
[c] R/S = (CAT activity with pRSV-Cat)/(CAT activity with pSV2-Cat).

model T-cell behavior, so we compared examples of these cell types with the primary and factor-dependent T cells described above. In contrast to the results obtained with growth factor-dependent cell lines, results from a panel of T-cell hybridomas and thymomas revealed only a slight preference, if any, for RSV over SV40 (Fig. 7 and Table 5). These modest R/S values (~2 to 5) were usually greater in unstimulated cells than in cells which had been stimulated overnight with phorbol ester and calcium ionophore, and in some cases stimulation resulted in R/S ratios that were slightly less than 1 (see, e.g., Jurkat cells in Table 5). The tumor cell lines in this panel were all rapidly and continuously growing, with cycle times quite comparable to those of the growth factor-dependent cells. Thus, a strong preference for RSV over SV40 is not a necessary consequence of rapid growth. On the other hand, all of the tumor cells also failed to match the R/S ratio of cortical thymocytes, even though some of them expressed certain aspects of cortical thymocyte phenotype. For example, the R1.1 thymoma appears to represent an immortalized cortical thymocyte by virtue of its high-level expression of the cortical thymocyte markers TL and terminal deoxynucleotidyltransferase (49b). The AKR thymoma lines 705, 629, and 728 also represent transitional thymocyte stages (44). All three lines lack surface TcR expression but differ in their expression of α- and β-chain mRNAs. Line 705 represents an immature thymocyte type, CD^- $CD8^-$ and lacking TcR α-chain mRNA. Line 629 is also $CD4^-$ $CD8^-$ but expresses both α- and β-chain mRNAs, and line 728 not only expresses TcR α- and β-chain mRNAs but is also double positive. Nevertheless, all three AKR lines have R/S ratios of about 1, and line R1.1 frankly prefers the RSV promoter (Table 5). Thus, the growth state of the transformed cells appears to preclude their use of the same promoter hierarchy as their presumed normal counterparts.

DISCUSSION

T-cell development proceeds through a complex series of intermediate stages which can be characterized, in part, by differential expression of stage-specific gene products. Thymocytes also differ in the extent to which they are capable of being induced to express genes which they do not constitutively transcribe. We have used a simple protocol, measuring the relative expression of viral promoter constructs, to demonstrate two points. First, both thymocytes and spleen T cells are easily transfected and can express levels of the CAT reporter gene which are readily detectable in a 5-h assay. Second, even within the T-cell lineage, commonly used positive control constructs are subject to sharp differential regulation, with their relative strengths shifting and even reversible as a function of developmental or growth state. The relative expression of the RSV and SV40 regulatory regions thus seems not to be dominated so much by the presence or absence of lymphoid-specific transcription factors per se as by sweepingly changeable patterns of general transcription factor expression that distinguish cortical thymocytes from their precursors, from their splenic progeny, and from cell lines derived from them.

Our results using DEAE-dextran to transfect murine splenic T cells are in good agreement with those of Cann et al. (9), who described conditions for transfecting human peripheral blood lymphocytes by electroporation. T cells from both sources are mature and predominantly in a resting state. As in our experiments, the human lymphocytes report-

edly required 3 days of prestimulation with the mitogen phytohemagglutinin plus IL-2 before transfection in order to express detectable CAT activity, even though they could take up DNA without prestimulation. According to the published data, these mitogen-stimulated human T cells had an R/S ratio of ~0.5, in reasonable agreement with the similar ratios of expression that we observed in murine ConA blasts. In our hands, however, the effect of prestimulation to confer competence could be separated from a further acute effect of TPA-A23187 induction after transfection to enhance expression. ConA blasts expressed CAT from all four viral promoters much better after such induction, even though the cells had already become activated and involved in the cell cycle. This finding suggests that a function directly controlled by Ca^{2+} flux and protein kinase C activation, and not merely an outcome of mitogenesis in general, influences exogenous DNA expression. While our data do not formally rule out a posttranscriptional effect, e.g., at the level of translation, there are well-established precedents for genes whose transcriptional activity in T cells shows this pattern of regulation, for example, the IL-2 and gamma interferon lymphokine genes (28). Thus, rate-limiting regulatory factors for all of these viral promoters may be controlled by Ca^{2+}-protein kinase C activation in T cells.

Our unexpected finding was how accessible murine thymocytes were for transfection and short-term expression of the exogenous DNA. Populations of thymocytes required no prestimulation in order to become competent to express these DNAs, even though TPA-A23187 treatment after transfection enhanced reporter gene expression as described above. Our data indicate that at least two developmentally distinct populations of thymocytes participate in this response. This conclusion is based on the fractionation behavior of the transfectable cells, coupled with the relative recoveries of pRSV-Cat expression versus pSV2-Cat expression in different thymocyte subsets. If this conclusion proves to be sound, then the two types of thymocytes that can be transfected are, respectively, immature (TcR^-) thymic blast cells and small cortical thymocytes.

The involvement of immature thymic blasts is not unexpected, as these are cells with high RNA contents, high protein synthesis rates, short cell cycle times in vivo, and excellent viabilities in vitro (8, 32, 40, 46, 51). This actively dividing minority of cells includes immediate precursors of most cortical thymocytes, but our data do not demarcate the precise stages of development that limit the transfectable state. SCID thymocytes, most of which are arrested at a $CD4^-$ $CD8^-$ IL-2 $receptor^+$ TcR^- stopping point before the final proliferative stage, include transfectable cells with an R/S ratio similar to that of normal thymic blast cells. While all are immature, it remains to be determined whether the cells in the SCID thymus that are transfectable are representative of the majority or merely a subset.

The immature blast cells do not represent all of the transfectable normal thymocytes, because while the blasts express RSV and SV40 promoters approximately equally, the total population shows at least 20-fold better activity with the SV40 promoter. Our fractionation data imply that the additional subset involved is that of the small cortical thymocytes. This is a surprising conclusion given the notorious fragility of these cells and their well-documented refractoriness in conventional responses to stimulation. The ability of small PNA^+ cells to be transfected is not due to their being in an actively cycling state, because on the basis of DNA content such cells are clearly in G_0/G_1 (5, 36, 49c), as are resting splenic T cells. On the other hand, several lines of evidence have long suggested that the resting state of cortical cells is at least an unusual one. They retain high concentrations of deoxynucleoside triphosphates, unlike most resting cells (14, 31), and high-level expression of proliferation-associated c-*onc* gene expression (54, 57, 58). Their persistent high-level expression of the DNA polymerase terminal deoxynucleotidyltransferase (38, 48) is also unexpected for resting cells. Thus, the unconventional physiological state of these cells may act to facilitate their processing of exogenous DNA for expression, in a way that other cells do efficiently only when in cycle.

While the MCF and Akv LTRs exhibit high promoter activity in ConA blasts, cortical thymocytes, and immature SCID thymocytes alike, the RSV LTR and SV40 early region respond very differently to different cellular contexts. Whereas ConA blasts and immature thymocytes utilize both comparably well (R/S from 0.3 to 3), RSV and SV40 expression patterns are divergently skewed in cortical thymocytes ($R/S \leq 0.05$) and IL-2-dependent, nonmalignant cell lines ($R/S \geq 10$). These reproducible differences, which are sustained with or without TPA-A23187 treatment, imply that the batteries of transcription factors available in cortical thymocytes and factor-dependent T cells are at least quantitatively different from those in T-lineage cells in other states. It is possible that the R/S phenotype of the factor-dependent lines is one which primary T cells also approach transiently, at times when they enter a stage of IL-2-dependent proliferation. For example, there was a trend toward higher R/S ratios in preparations of ConA blasts in which the more IL-2-responsive $CD8^+$ cells (27) had most outgrown the $CD4^+$ cells. However, our results clearly show that high R/S ratios are not a consequence of proliferation per se. Dividing thymic blast cells and a wide range of tumor cell lines were largely indifferent to the two promoters. T cells proliferating in a factor-dependent manner therefore appear to possess a distinctive signature of regulatory proteins, leading to distinctive promoter preferences.

Cortical thymocytes appear at the opposite pole of regulatory physiology, with a virtual inability to utilize the RSV LTR in spite of robust expression of the SV40 early region. Definition of the basis of the RSV expression defect must await fuller characterization of the transcription factors that normally act on this LTR. SV40 regulatory factors, on the other hand, are well defined, and it has recently become possible to analyze thymocytes directly for their expression. While such studies are in a very early stage, the initial results appear quite compatible with those of our functional studies. One general transcription factor that is required for efficient expression of the SV40 early promoter is Sp1 (18, 21, 30). It is interesting that thymocytes of 4- to 5-week-old mice reportedly express higher levels of Sp1 mRNA than do any other of a series of mouse tissues examined (52). The poor expression of the enhancerless SV40 derivative pUC18-Cat, which retains all six Sp1 sites of the normal SV40 promoter, demonstrates that Sp1 protein-DNA interactions are not sufficient for high-level transcription; however, high levels of Sp1 are certainly consistent with the relatively strong utilization of the intact SV40 promoter by the cortical thymocytes. At least two additional binding sites in the SV40 enhancer are particularly likely to be engaged by cortical thymocyte proteins, namely, the octamer motifs associated with the twin *Sph*I sites and the AP-1 sites immediately downstream from them. Direct analysis of the binding proteins in cortical thymocyte nuclear extracts shows abundant constitutive octamer-binding activity (11a, 45). Furthermore, these cells can readily be activated to express a strong

binding activity for AP-1-like sites, as well as another inducible factor, CD28RC, which may bind to a sequence similar to the target of AP-3 (11a). Many more factors remain to be measured, but it seems likely that the bias of cortical thymocytes toward expression of the SV40 early region promoter will soon be substantiated in molecular terms.

In contrast to the SV40 and RSV regulatory regions, the MCF and Akv LTRs are powerful promoters in both thymocytes and ConA-treated spleen cells. One possible explanation for the significantly greater activity of the MCF and Akv LTRs would be the ability of certain enhancers and promoters to function in a species-specific manner (2, 34). The recent observation that the activity of the TFIID transcription factor that binds to promoter sequences can be species dependent in vivo (16, 22) may partly account for this species specificity. Hence, the fact that the MCF and Akv promoters are derived from murine retroviruses may contribute to their increased expression relative to the RSV and SV40 promoters, which are of avian and simian origin, respectively. However, much remains to be learned about the specific participants in protein-DNA and protein-protein interactions on these LTRs. It has been shown that the MCF and Akv enhancers contain discrete protein-binding sites that have been implicated in the TPA responsiveness of the LTR of the Moloney murine leukemia virus, which is related to the MCF and Akv viruses (56). Although these protein-binding sites may be important for the increase in transcriptional activity detected for the MCF and Akv LTRs, it is clear that they are not sufficient for this response, since these viral enhancers are unable to activate the SV40 promoter in the MS1 and AS1 constructs. Elucidation of such regulatory interactions will be needed to explain the broad range of T-cell types in which these LTRs are active. In contrast to our earlier observation that the MCF LTR is transcriptionally more active than the Akv LTR in T-cell lines (62), both LTRs were equally active in PNA^+ thymocytes. It is thought that the MCF but not the Akv virus infects and transforms a cycling subset of thymic cortical cells, i.e., the cortical blast cell (12, 43). Based on the mechanism of insertional mutagenesis by the MCF LTR in thymic tumors (15, 17), it has been hypothesized that the MCF LTR would be more active than the Akv LTR in such a population of cortical cells. One reason we may not have detected any difference in transcriptional activity between these two LTRs is that this blast cell population represents only a small percentage of the PNA^+ cells, and differences in CAT activity in this subset would be obscured by the unexpectedly high expression of both LTRs in the majority class of cortical cells. A definitive test of the hypothesis thus awaits isolation of the critical cortical blast cell subset and a direct comparison of the MCF and Akv LTRs in that cellular context.

The CAT constructs used in this study were not intended to be developmentally stage specific. While lineage-specific elements have been implicated in their regulation, they were used here as examples of positive control constructs against which regulatory sequences implicated in lymphocyte responses could be compared. The results of these experiments nevertheless show that within the T-cell lineage, changes in the utilization of general transcription factors can be extensive enough to allow common viral promoters to serve as probes of developmental state and mitogenic mechanism.

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