Transcriptional Regulation of the Interleukin-2 Gene in Normal Human Peripheral Blood T Cells

CONVERGENCE OF COSTIMULATORY SIGNALS AND DIFFERENCES FROM TRANSFORMED T CELLS*

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To study transcriptional regulation in normal human T cells, we have optimized conditions for transient transfection. Interleukin-2 (IL-2) promoter-reporter gene behavior closely parallels the endogenous gene in response to T cell receptor and costimulatory signals. As assessed with mutagenized promoters, the most important IL-2 cis-regulatory elements in normal T cells are the proximal AP-1 site and the NF-κB site. Both primary activation, with phytohemagglutinin or antibodies to CD3, and costimulation, provided by pairs of CD2 antigens or B7-positive (B cells) or B7-negative (endothelial) accessory cells, are mediated through the same cis-elements. Interestingly, the nuclear factor of activated T cell sites are much less important in normal T cells than in Jurkat T cells. We conclude that IL-2 transcriptional regulation differs in tumor cell lines compared with normal T cells and that different costimulatory signals converge on the same cis-elements in the IL-2 promoter.

The initiation of an immune response involves interaction between foreign antigenic peptide bound to major histocompatibility complex (MHC) molecules on the antigen-presenting cell (APC) and a cognate antigen receptor on the T cell (1). Under physiological conditions, specific antigen-MHC complexes are usually limiting, and T cells require additional costimulatory signals to be fully activated (2). The major source of such signals appears to be direct interaction of cell surface ligands on the T cell and the APC.

T cells that receive the correct combination of T cell receptor (TCR)-mediated and costimulatory signals enter the cell cycle, express activation antigens such as CD69 and CD25, and begin synthesis of several cytokines, including the T cell autocrine/paracrine growth factor interleukin-2 (IL-2) (2, 3). IL-2 is an essential factor required for progression of newly activated T cells from G1 to S phase, and the quantity of IL-2 produced is a major determinant of whether an effective response can be generated. Moreover, inadequate IL-2 synthesis can lead to T cell death or induction of a state of unresponsiveness known as anergy (4).

Numerous T cell surface molecules have been suggested as mediators of costimulatory signals, the best characterized being CD2, interacting primarily with CD58 (LFA-3) and CD59 on the APC (5–8), and CD28, interacting with CD80 (B7-1) and CD86 (B7-2/B70) on the APC (9–13). CTLA-4 is a second T cell molecule that interacts with the same ligands as CD28 and may also mediate costimulatory signals (14), although this has been disputed (15, 16). However, costimulatory activity is not limited to these molecules (17, 18). Both the CD2 and CD28 pathways of costimulation are thought to increase the level of IL-2 transcription in human T cells, although this may not be the case for CD28 in murine T cell clones (19). CD28-mediated signals may additionally stabilize IL-2 mRNA and affect posttranscriptional nuclear processing (19, 20).

The IL-2 gene is not actively transcribed in resting T cells. Transcription of IL-2 can be detected as early as 40 min after activation, leading to peak levels of mRNA around 6 h and a return to near zero levels by 12–18 h (2, 5). Approximately 300 base pairs of the IL-2 promoter are sufficient to confer cell-specific inducible expression to reporter gene constructs (21), although other regulatory sequences may lie outside of this region (22). Within these 300 base pairs, several transcription factor-binding sites have been identified as positive regulatory elements in tumor T cells (see Table I), including proximal and distal specific sequences for the nuclear factor of activated T cells (NFAT) (21, 23, 24) and proximal and distal sequences for AP-1 (25, 26), for NF-κB (27, 28), for NIL-2A (29), for CD28-activated factors (30), and for octamer factors (31). An additional AP-1 site has also recently been identified just downstream of the dNFAT sequence (25), which we have designated as NFAP-1 as it appears to be functionally a part of the "NFAT" binding sequence. Also, a binding site for SP1 and EGR-1 has been identified immediately upstream of the distal NFAT site (32). Many of these sites vary from consensus sequences in other genes, and it appears that these differences account, at least in part, for the T cell-specific expression of the IL-2 gene (33).

Activation of T cells through the TCR/CD3 signaling pathway leads to the activation of transcription factors specific to several of these sites. It has been proposed that costimulatory signals increase transcription by either 1) altering the composition of transcription factors that bind to sites targeted by TCR/CD3-derived signals or 2) inducing new factors that bind to novel combinations of sites. We have previously presented evidence that human umbilical vein endothelial cell (EC) costimulation modifies the composition of the AP-1 complex binding to the proximal AP-1 (pAP-1) site in mitogen-activated T cell...
IL-2 Transcription in Peripheral Blood Lymphocytes

Preparation and Transfection of T Cells—Normal human peripheral blood mononuclear cells (PBMC) were prepared by centrifugation of whole blood or lymphocyte separation medium according to the manufacturer’s instructions (Littton Bionetics, Kensington, MD). Blood was obtained by venipuncture of normal healthy donors. Cells were cultured for 19.5–20 h in RPMI 1640 medium containing 10% fetal calf serum, antibiotics (Life Technologies, Inc.), and 50 μm 2-mercaptoethanol with 1 μg/ml PHA-L (Sigma) at 3 x 10³ cells/ml to induce transfection competence. After washing, cells were suspended in fresh lymphocyte medium at 2 x 10⁷/ml. Aliquots of 0.25 ml were electroporated in a BioRad Gene Pulser at 250 V and 960 microfarads at room temperature in the presence of 60 μg/ml reporter gene DNA (see below). Where different mutant promoters were being compared in a single experiment, pXGH (growth hormone driven by the metallothionein promoter) or pCMV-β-gal (β-galactosidase driven by the cytomegalovirus promoter) (10 μg/ml) was added to control for transfection efficiency. Normalizing for transfection efficiency was not necessary when a single promoter (usually wild-type) was being assayed under different conditions; here separate transfections were pooled before use. The gap width of the cuvettes was 0.4 cm, which resulted in a τ of 45–50 ms. Jurkat cells, treated with PHA in parallel with PBMC, were electroporated under the same conditions, except that a volume of 0.5 ml was used, which reduced τ to 20–25 ms. After electroporation, cells were immediately removed and added to 10 ml of medium, washed, and resuspended in fresh medium. Cells were plated (see below) after resting for 2 h at 37°C.

Sorting of Cells by FACS—PBMC were electroporated as described above and then incubated with antibodies directly conjugated with fluorescein isothiocyanate or phycoerythrin for 20 min on ice in phosphate-buffered saline, 0.1% mouse serum. After washing, cells were resuspended in RPMI 1640 medium, 0.5% fetal calf serum, and aliquots were sorted on a FACScan cell sorter (Becton Dickinson, Mountain View, CA). Abs used were as follows: anti-CD4-fluorescein isothiocyanate and anti-CD8-phycoerythrin against T cells (Sigma), anti-CD19-phycoerythrin against B cells, and anti-CD14-fluorescein isothiocyanate against monocytes (the latter two from Becton Dickinson).

Detection of Reporter Genes—For detection of luciferase and β-galactosidase activity, plates were spun down, and 150 μl of medium was removed (this was saved for IL-2 bioassay; see below). To the remaining 50 μl containing the cells was added 100 μl of 1.5 × reporter lysis buffer (Promega, Madison, WI). This amount of medium did not interfere significantly with the subsequent assays. Light output from luciferase was assayed in a luminometer (Bio-Tek Instruments, Winooski, VT) by using a 10 s integration time using a 96-well plate. After light output from transfection and 1.5 × reporter lysis buffer was assayed, the plate was washed and 100 μl of reporter lysis buffer were added to each well. Light output was then measured using a 96-well plate. The final concentration of light output from luciferase was measured using a commercially available kit (Promega).

Culture of Endothelial Cells and B Cells with T Lymphocytes—Human umbilical vein EC were plated in fibronectin-coated round bottom 96-well plates and grown to confluence in medium M199 containing 20% fetal bovine serum, antibiotics, endothelial cell growth supplement, and heparin as described previously (34). B cells (1 x 10⁴/well) were plated in lymphocyte medium and plated into round bottom 96-well plates. After 16 h of transfection and with 1.5 × reporter lysis buffer were added to EC, to B cells, or to fibronectin-coated wells in lymphocyte medium. This number of T cells represents a ratio of ~30:1 over APC. The same number of Jurkat T cells was added to similarly coated wells. PHA was used at 5 μg/ml. Anti-CD3 mAb OKT3 was bound to anti-mouse Ig-coated beads according to the manufacturer’s instructions (Dynal, Inc., Great Neck, NY). The final concentration of bead-bound mAb in the wells was ~0.5–1 μg/ml. Phorbol ester (PMA; Sigma) was used at 10 ng/ml and ionomycin at 200 ng/ml. The stimulating anti-CD2 mAbs CB6 and GD10 (a gift of Chris Benjamín, Biogen Inc., Cambridge, MA) were purified from ascites and used at 1 μg/ml. Anti-CD2 mAb TS2/18 and anti-LFA-3 mAb TS2/9 (a gift of Tim Springer, Center for Blood Research, Boston) were also used for transfection of the B cells, and used at 10 μg/ml. Anti-CD28 mAbs 3D10 (a gift of Gary Gray, Repligen, Cambridge, MA), 9.3 (a gift of J. Ledbetter, Bristol-Myers Squibb, Seattle), and L293 (Becton Dickinson) were used at 1 μg/ml. CTLA-4-Ig fusion protein, control Ig fusion protein, mAb 9.3 Fab fragments, and control Fab fragments were also gifts of Gary Gray and were used at 10 μg/ml.

IL-2 Bioassay—The concentration of IL-2 in the medium was determined by HT-2 bioassay as described previously (34). HT-2 cells were a gift of A. Abbas (Brigham and Women’s Hospital, Boston). At least three serial dilutions of each supernatant were tested, and results are shown as mean ± S.D. None of the reagents used were found to significantly affect cell survival.

Generation of IL-2 Promoter Mutations—A 600-base pair HindIII fragment of the human IL-2 promoter (a gift of G. Crabtree, Stanford University, Stanford, CA) was subcloned into the luciferase reporter plasmid pGL2-Enhancer (Promega), and the orientation was confirmed by restriction enzyme mapping. Three base pair mutations were selected that altered nucleotides known to be critical for nuclear factor binding (see Table I). Reaction conditions were as follows: 100 pg of template, 20 pmol of each primer, 2 mM MgCl₂, and 200 μM each dNTP in 1 x polymerase buffer with 1 unit of Taq polymerase in a final volume of 50 μl; initial denaturation at 95°C for 1 min; amplification for 30 cycles (95°C for 30 s, 50–53°C depending on primer pair) for 30 s, and 72°C for 1 min) and final extension at 72°C for 10 min. Overhangs at the 3'-end, generated by Taq terminal transferase activity, were removed by treatment with T4 DNA polymerase (46). Amplification products were separated on 1.5% low melting agarose gels (SeaPlaque LM, FMC Corp. BioProducts, Rockland, ME); bands were cut out and purified; and the two halves of the SnaBI site were ligated by the ligation of the two ends of PCR were ligated into the SnaBI site of SnaBI-digested pGL2-Enhancer. The promoters were subcloned back into pGL2-Enhancer, and the sequences were confirmed by dye sequencing (U. S. Biochemical Corp.). Large-scale DNA preparations were performed by selective absorption to resin columns according to the manufacturer’s instructions (QIAGEN Inc., Chatsworth, CA). Experiments comparing different promoter mutations were performed with at least two independent plasmid preparations. Experiments using the wild-type promoter were repeated with more than five independent plasmid preparations.

Electrophoretic Mobility Shift Assay—Probes of 18–30 base pairs

were obtained by venipuncture of normal healthy donors. Cellswere cultured for 19.5–20 h in RPMI 1640 medium containing 10% fetal calf serum, antibiotics (Life Technologies, Inc.), and 50 μm 2-mercaptoethanol with 1 μg/ml PHA-L (Sigma) at 3 x 10³ cells/ml to induce transfection competence. After washing, cells were suspended in fresh lymphocyte medium at 2 x 10⁷/ml. Aliquots of 0.25 ml were electroporated in a BioRad Gene Pulser at 250 V and 960 microfarads at room temperature in the presence of 60 μg/ml reporter gene DNA (see below). Where different mutant promoters were being compared in a single experiment, pXGH (growth hormone driven by the metallothionein promoter) or pCMV-β-gal (β-galactosidase driven by the cytomegalovirus promoter) (10 μg/ml) was added to control for transfection efficiency. Normalizing for transfection efficiency was not necessary when a single promoter (usually wild-type) was being assayed under different conditions; here separate transfections were pooled before use. The gap width of the cuvettes was 0.4 cm, which resulted in a τ of 45–50 ms. Jurkat cells, treated with PHA in parallel with PBMC, were electroporated under the same conditions, except that a volume of 0.5 ml was used, which reduced τ to 20–25 ms. After electroporation, cells were immediately removed and added to 10 ml of medium, washed, and resuspended in fresh medium. Cells were plated (see below) after resting for 2 h at 37°C.

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Transfection of Normal Peripheral Blood Lymphocytes—To investigate the regulation of IL-2 transcription in normal human T cells, we have developed a reproducible assay based on transient transfection by electroporation of IL-2 promoter-luciferase reporter gene constructs. The technique is sufficiently sensitive to detect luciferase activity in as few as 8 x 10^6 cells (40 μl of a 150-μl lysate), allowing routine analysis of microwell cultures.

Circulating human T cells are not transfectable. This has led most investigators to work with T cell tumor lines, cells that may not accurately reflect normal T cell behavior (see below). However, we have found that the T cells in a freshly isolated population of PBMC, upon stimulation with a concentration of PHA (1 μg/ml) that is insufficient to cause significant IL-2 secretion, pass through a window of transfection competency. The mitogen PHA polyconally activates T cells by binding with high affinity to the TCR, but not to CD3, and inducing calcium fluxes in a similar manner to anti-CD3 or anti-TCR mAb (47). Thus, presentation of antigen by MHC molecules is bypassed. In the two separate experiments shown in Fig. 1, PBMC were transfected by electroporation as described under “Experimental Procedures” at the indicated times after PHA stimulation; luciferase was assayed in all cases after restimulation for 6 h. Transcription peaks in a similar manner to anti-CD3 or anti-TCR mAb (47). The Transfected IL-2 Promoter Can Only Be Activated in T Cells—An important consideration in interpreting our data is that we transfect a mixed population of cells that, in addition to T cells, contains monocytes, B cells, natural killer cells, and dendritic cells. We have found that highly purified T cells cannot be made transfection-competent by treatment with PHA alone (data not shown). Therefore, to determine which cell types in the unfractionated PBMC population are expressing the IL-2 promoter-luciferase construct under the stimulation conditions we use, we sorted cells by FACS after transfection using directly conjugated mouse anti-human mAb specific for CD4^+ T cells, CD8^+ T cells, B cells, and monocytes. Sorting produced populations of >99.5% purity. When assayed for luciferase activity, only CD4^+ T cells and, to a lesser extent, CD8^+ T cells expressed significant activity in response to PHA plus PMA. An equal number of monocytes or B cells (10-fold more than are present in unfractionated peripheral blood mononuclear cell preparations) expressed little to no activity (Fig. 3). In additional experiments, we confirmed the complete

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**RESULTS**

To determine the time course of transcription mediated by the IL-2 promoter in transfected normal T cells, we transfected cells as described, stimulated them with optimal doses of PHA and PMA, and then assayed luciferase activity and secreted IL-2 over the next 48 h. As shown in Fig. 2, onset of transcription is rapid, being easily detectable at 2 h. Transcription peaks between 4 and 8 h and declines rapidly to ~20% of peak levels by 18 h and then more slowly to near basal levels by 48 h. These kinetics are consistent with previously reported kinetics of mRNA appearance following the activation of resting human blood T cells (5) and parallel the behavior of IL-2 promoter-reporter genes in the widely used T cell tumor line, J urkat (data not shown and Ref. 2). More important, no transcription was detectable in transfected PBMC in the absence of additional stimulation, despite the prior exposure to low concentrations of PHA.

Secreted IL-2 was measured as an indicator of the activity of the endogenous gene in the medium of the same cultures. No secreted IL-2 is detectable in the absence of stimulation, indicating that the native IL-2 gene is not active as a result of the prior manipulations to render the cells transfection-competent. IL-2 is first measurable in the medium at ~3–6 h and then rapidly increases over the next 20 h. Levels begin to plateau after ~24 h. These data are also consistent with previously published reports of resting T cells (34, 48).

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**Fig. 1.** Optimal time for transfection of normal T cells. Cells were cultured with 1 μg/ml PHA for the indicated times and then transfected with the wild-type IL-2 promoter-luciferase reporter plasmid (60 μg/ml) as described under “Experimental Procedures.” Cells were restimulated with PHA (5 μg/ml) and PMA (50 nM) and harvested 6 h later for assay of luciferase. Means of triplicate determinations are shown for luciferase expression. Standard errors were <10% of the mean. Data points indicate relative light units: □, Experiment 1; △, Experiment 2. Each curve is an independent experiment.

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**Fig. 2.** Time course of IL-2 promoter activity and IL-2 synthesis in transfected normal T cells. Normal T cells were transfected with the wild-type IL-2 promoter-luciferase reporter plasmid (60 μg/ml) as described under “Experimental Procedures” and cultured in the presence of PHA (5 μg/ml) and PMA (50 nM) for the indicated times before harvest of the supernatant for IL-2 bioassay and of the cells for luciferase assay. Means and standard errors are as described for Fig. 1. No luciferase activity above background levels or IL-2 was detected in the absence of PHA plus PMA. Data are representative of one of three experiments with similar results. □, relative light units; △, units/milliliter IL-2.
absence of luciferase activity in the total non-CD4+/CD8+ cell population (data not shown). Luciferase is detectable in this population when the SV40 promoter is used to drive reporter gene expression (pGL2-Con), indicating that some cells in the non-T cell population are transfectable (data not shown). Luciferase is detectable in the population of cells used in our experiments, only CD4+ T cells and, to a lesser extent, CD8+ T cells express the transfected IL-2 promoter-luciferase construct.

Response of the Transfected Cells to Various Stimulators and Costimulators—We next investigated the response of the IL-2 promoter in normal T cells to various primary (T cell receptor-directed) and secondary (costimulatory) signals in order to determine if transfected cells behave similarly to normal untransfected cells. It is well established that in normal T cells, the IL-2 promoter responds well to a combination of ionomycin, a calcium ionophore, and PMA, but only weakly to either agent alone (48). We have confirmed that the transfected promoter behaves similarly (Table I, Experiment 1) and that it parallels the response of the endogenous gene in these cells, measured as secreted IL-2. As we reported above, both the lectin PHA and anti-CD3 mAb OKT3, which activate T cells via the T cell receptor, also activate transcription of the reporter gene (Table I, Experiment 2). PHA, which alone provides an exceptionally strong signal that in many cases bypasses the need for a co-stimulator, activated the IL-2 promoter almost 4-fold above background levels and induced significant IL-2 synthesis. OKT3 alone, however, only weakly activated the promoter and did not induce IL-2 synthesis. CD28 ligation on T cells provides strong costimulatory signals, but antibody to CD28 alone only weakly activated the promoter and again did not induce significant IL-2 synthesis. However, anti-CD28 mAb synergized with OKT3 to stimulate both IL-2 transcription and IL-2 synthesis. Finally, it has been previously shown that pairs of anti-CD2 mAbs can activate T cells and induce IL-2 synthesis in the absence of T cell receptor ligation. As shown in Table I (Experiment 2), such mAbs stimulate IL-2 promoter-dependent transcription in transfected normal cells.

Effect of B7-positive and B7-negative Accessory Cells on IL-2 Promoter-dependent Transcription—We next examined the effect of different accessory cell populations on costimulation of IL-2 promoter-dependent transcription and IL-2 synthesis in normal T cells. We compared B cells, which express CD28 ligands, with EC, which do not. Three B cell lines were tested: JY, an Epstein-Barr virus-transformed line; Raji, an Epstein-Barr virus-positive Burkitt's lymphoma line; and BJAB, an Epstein-Barr virus-negative Burkitt's lymphoma line. All three lines expressed CD28 ligands, and results were similar, except that JY and BJAB cells were generally better costimulators of IL-2 synthesis than were Raji cells; data from experiments with JY and BJAB cells are presented. EC were tested from multiple donors with similar results, and representative results are shown.

As shown in Fig. 4A, both B cells and EC augmented reporter gene transcription in PHA-activated normal T cells above the level induced by PHA alone. Over several experiments, augmentation ranged from 2- to 7-fold, with EC and B cells augmenting to approximately the same level. Expression of the endogenous gene was also augmented, but B cells seemed to be much more effective at augmenting secreted IL-2 than were EC (Fig. 4B). Both B cells and EC also costimulated T cells purified by FACS. In this case, little or no luciferase or secreted IL-2 was detectable in the absence of accessory cells. IL-2 promoter-dependent transcription was detectable in both highly purified CD4+ and CD8+ T cells stimulated by accessory cells, and both cell types secreted IL-2. CD4+ T cells were severalfold more responsive than CD8+ T cells (data not shown).

To identify the surface molecules involved in costimulation by B cells and EC, we used blocking antibodies and fusion proteins. mAb to CD2 blocked transcription and IL-2 secretion induced by B cells or EC (Fig. 4, A and B). Consistent with our previous findings (5), anti-CD2 mAb could not completely inhibit EC costimulation. Blocking of B cell costimulation with anti-CD2 mAb was also incomplete, ranging from 40 to 90%. Most experiments were performed with anti-CD2 mAb TS2/18;
however, we obtained identical results using anti-CD2 mAbs 35.1 and TS1/8 or anti-LFA-3 mAb TS2/9 (data not shown).

To investigate the role of CD28-mediated signals in this system, we used CTLA-4-Ig fusion protein, which binds to B7-1 and B7-2 and blocks interaction with their ligands, CD28 and CTLA-4. Consistent with our previous data demonstrating the absence of CD28 ligands on human EC and the lack of effect of CTLA-4-Ig on CTLA-4 mAb Fab fragments on EC costimulation of IL-2 secretion (49), CTLA-4-Ig did not block EC costimulation of IL-2 promoter-dependent transcription (Fig. 4A). Similarly, this reagent did not block IL-2 secretion in response to EC. In sharp contrast, however, CTLA-4-Ig was very effective at blocking the costimulatory effects of CD28 ligand-bearing B cells on both transcription (Fig. 4A) and IL-2 secretion (Fig. 4B). Indeed, CTLA-4-Ig completely inhibited the augmented secretion of IL-2 in response to B cell costimulation. We have found substantially similar, although somewhat more variable, results using anti-CD2 mAbs TS2/18 (data not shown).

Analysis of IL-2 Promoter Elements in Human Peripheral Blood T Cells—In the next series of experiments, we examined the function of different sequence elements in the IL-2 promoter by transfection of normal human T cells. Specific mutations were generated in several of the recognized cis-regulating sites of the human IL-2 promoter by a recombinant PCR technique (Table II). Three base pair mutations were selected that altered nucleotides known to be critical for nuclear factor binding. This approach allows sequences to be altered without affecting the spacing between individual sites. The result of each mutagenesis reaction was confirmed by sequencing, and the functional significance of the mutation was tested by annealing the inside primers and using this double-stranded probe to compete for protein binding with the wild-type sequence in an electrophoretic mobility shift assay. In all of the mutant constructs tested, the base pair changes we introduced were found to eliminate binding of the cognate transcription factors in vitro. It should be noted, however, that lack of binding in vitro does not necessarily rule out binding of factors in vivo. Representative data for the NF-κB site are shown in Fig. 5. In this experiment, nuclear factor bound to the wild-type NF-κB probe and was competed for by an excess of wild-type, but not mutant, probe.

Each of the mutant promoters was cloned upstream of the luciferase reporter gene and transfected into normal T cells along with either pXGH (a growth hormone-expressing plasmid) or pCMV-β-gal (a β-galactosidase-expressing plasmid) to normalize for transfection efficiency. Transfected cells were then stimulated with PHA or with PHA plus PMA and harvested 12 h later. Levels of secreted IL-2 produced by the transfected cells varied by <10% between the different constructs (data not shown). As shown in Fig. 6, mutations in several sites reduced transcription in PHA-activated normal T cells, including the dNFAT, NF-κB, and pAP-1 sites. Over several experiments, the pAP-1 site was consistently the most important single site. Mutation of this element reduced transcription by 85–95%. Mutating the NFAP-1 or dAP-1 sites generally had small effects (0–30% reduction), and in several experiments, mutating the dAP-1 site had no effect.

Interestingly, we noted a striking difference between normal T cells activated with PHA plus PMA and those activated with PHA alone. Specifically, the NF-κB site appeared to be much more important in the absence of the strong protein kinase C activator PMA (Fig. 6). We have repeated this experiment using normal T cells activated with anti-CD3 mAb OKT3 and find the same pattern as with PHA alone. Under these conditions, the pAP-1 and NF-κB sites generally had small effects (0–30% reduction), and in several experiments, mutating the dAP-1 site had no effect.

IL-2 Promoter cis-Elements Targeted by Different Accessory Cells—We have established that two different accessory cells, B cells and EC, costimulate T cells through different surface molecules. EC engage T cell CD2 via LFA-3 (and/or CD59), but do not stimulate through CD28. Conversely, B cells stimulate T cells through CD2 and CD28 (and/or CTLA-4). Clearly, both

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accessory cells may also stimulate through other pathways not examined in this report. We next wished to determine whether these different accessory cells activate the IL-2 promoter via the same combination of promoter cis-elements or through novel combinations. Peripheral blood T cells were transfected with the various mutant promoter constructs and then stimulated in the presence of B cells or EC. As shown in Fig. 7A, mutations of the various cis-elements affect B cell and EC costimulation equally. Specifically, mutations in the pAP-1 site or the NF-κB site were most effective in reducing transcription, with important contributions from the pOCT and NFAT sites. The pNFAT site was consistently more important than the dNFAT site, although mutations in both were additive. Interestingly, mutation of the CD28RE did not affect costimulation by the CD28 ligand-positive B cells, an unexpected result given the previous identification of this site as the target of CD28-mediated signaling. Over several experiments, using B cells as costimulators, we consistently failed to see a reduction in transcription as a result of mutating this site.

Finally, we repeated these experiments to compare defined costimulatory antibodies with accessory cells. We used an activating pair of anti-CD2 mAbs or an anti-CD28 mAb in conjunction with an anti-CD3 mAb (Fig. 7B) and compared these to EC and B cells (Fig. 7C). Again, the same combination of cis-regulatory elements was critical in each case. The pAP-1 and NF-κB sites were most important for CD2-, CD28-, or accessory cell-mediated costimulation. The pOCT and NFAT sites also contributed, along with the NFAP-1 site. Interestingly, although the CD28RE mutation did not affect CD2-, B cell-, or EC-mediated costimulation, it did have a variable effect on anti-CD28 mAb-mediated transcription: in three of five experiments, we saw a 25–50% reduction in transcription from the mutant CD28RE promoter compared with the wild-type promoter (Fig. 7B and data not shown).

Comparison of Normal T Cells with Jurkat Tumor Cells—The Jurkat T cell tumor line is the most widely used model for study of human transcriptional regulation of IL-2. Typically, these cells are stimulated with PHA plus PMA; activation in the absence of PMA is ineffective. In the experiments reported here, we also pretreated the Jurkat cells with low doses of PHA so that the conditions were comparable to those used for normal cells, although pilot experiments established that this treatment did not affect the observed results. Results with Jurkat T cells differed in several respects from those observed with normal T cells examined in parallel (Fig. 8). Most striking, mutation of the dNFAT and NFAP-1 sites was consistently much more effective at reducing transcription in Jurkat cells (up to 75%) than were these same mutations in normal T cells (0–50%). These results are consistent with previously published reports detailing the importance of the dNFAT site in Jurkat cells (21, 27, 50). Mutation of the NF-κB site is without effect, but this may be attributable to the need to use PMA to activate the cells (see above).

DISCUSSION

Transfected normal human T cells provide a sensitive and physiologically relevant model for studying cytokine transcription. We have used this system to analyze the regulation of IL-2 synthesis and find that the human IL-2 promoter is inducible in these cells and responds to the same primary and costimulatory signals as the endogenous promoter. Furthermore, we find that the regulation of IL-2 transcription in normal T cells differs from that in the tumor cell line Jurkat. Finally, we have shown that transfected normal human T cells respond to costimulation by different APC and that B cells and EC signal through different costimulatory ligands, but target the same cis-acting elements in the IL-2 promoter.
In the experiments described here, we have transfected a mixed population of cells. However, by use of FACS of transfected cells, we have determined that only T cells express the luciferase reporter gene and that only T cells secrete IL-2. CD4\(^+\) T cells are severalfold more active in this respect than CD8\(^+\) T cells. Thus, in this system, the IL-2 promoter is highly T cell-specific.

A potential limitation of these studies is that we must culture normal T cells with 1 \(\mu\)g/ml PHA to induce transfection competence. Under these conditions, there is no significant IL-2 production. IL-2 is required for progression of T cells from G\(_1\) to S phase (2). This treatment, therefore, probably moves the T cells from G\(_0\) to G\(_1\). However, several observations demonstrate that transfected normal T cells still respond similarly to freshly isolated resting T cells. 1) Once transfected, the cells do not secrete IL-2 or transcribe the reporter gene unless further activated with mitogen. 2) The kinetics of the transcriptional response parallel those of the endogenous IL-2 gene in resting T cells. 3) OKT3 signals are weak and must be costimulated by anti-CD28 mAb to activate optimal IL-2 transcription. 4) The cells can receive costimulation from APC. 5) Costimulatory WT and mutant (MT) IL-2 promoter-luciferase reporter plasmids (50 \(\mu\)g/ml) along with pXGH or pCMV.-\(\beta\)-gal (10 \(\mu\)g/ml) as described under “Experimental Procedures” and cultured for 12 h before harvest. A. Transfected T cells were cultured with 5 \(\mu\)g/ml PHA either alone or in the presence of EC (1 \(\times\) 10\(^4\) well) or JY cells (1 \(\times\) 10\(^4\) well). Data are representative of one of six similar experiments. B. Transfected T cells were cultured either alone or with anti-CD2 mAbs CB6 and GD10 (1 \(\mu\)g/ml) or anti-CD3 mAb OKT3 (0.5-1 \(\mu\)g/ml) plus mAb 9.3 (1 \(\mu\)g/ml). Data are representative of one of two similar experiments. C. Transfected T cells were cultured either alone or with OKT3 (0.5–1 \(\mu\)g/ml) in the presence of EC (1 \(\times\) 10\(^4\)well) or JY cells (1 \(\times\) 10\(^4\)well). Data are representative of one of two similar experiments. Shown are means ± S.E. of triplicate determinations. IL-2 in the medium did not vary by more than 10% between pools of cells transfected with each of the constructs. RLU, relative light units.

FIG. 7. Effect of nuclear factor-binding site mutations on IL-2 transcription in normal T cells costimulated by EC, J Y B cells, or costimulatory antibodies. Cells were transfected with the wild-
lution can be blocked by mAb to CD2 or fusion proteins that bind CD28 ligands.

The IL-2 promoter contains numerous transcription factor-binding sites. Several of these have been shown by genomic footprinting to bind proteins, and some have been shown to be functional by transfection of mutant reporter gene constructs (21, 24, 28, 35, 39, 41, 50). However, nearly all of these studies have been done in tumor cell lines, with the assumption that gene regulation in these cells would be identical to that in normal T cells, and there is little or no direct information as to which sites are relevant for IL-2 transcription in normal non-transformed T cells. A step toward analysis of the IL-2 promoter in vivo has been made by the generation of transgenic mice with the IL-2 promoter driving lacZ expression (51).

This study suggests that differences between normal cells and tumor cells do exist. In Jurkat cells, for example, we confirm that the dNFAT site is critical for inducible transcription regulated by the IL-2 promoter (50). The nearby sequence we have designated as NFAP-1 was equally important. In normal T cells, however, these sites were far less important. We have found that the pNFAT site is more important than the dNFAT site and that the NF-κB and PAP-1 sites are quantitatively most important. Mutation of either element almost completely blocked transcription in PHA-stimulated normal T cells. The recent generation of an NF-κB p50 knockout mouse suggests that the NF-κB site may be important in vivo as T cells from these mice failed to proliferate to mitogenic signals, suggesting a defect in the generation of IL-2 (52). Surprisingly, we found a difference in the importance of the NF-κB site in normal T cells in the presence and absence of PMA. Specifically, in the presence of PMA, mutation of the NF-κB site reduced transcription by ∼50%. However, when normal T cells were activated in the absence of PMA, by PHA, by OKT3 plus anti-CD28 mAb, or by pairs of anti-CD2 mAbs, mutation of the NF-κB site consistently reduced transcription by 75–90%. Most studies of Jurkat cells have used PHA plus PMA or ionomycin plus PMA to activate the cells. This suggests that studies of tumor cells requiring the use of nonphysiologic activators such as PMA may additionally complicate interpretation of results.

APC activate T cells by presenting antigen in the context of self-MHC molecules and providing costimulatory signals such as those mediated by CD2 and CD28. We compared B cells, bone marrow-derived “professional” APC expressing B7, with B7-negative EC, which have been described as “semiprofessional” APC for their ability to costimulate IL-2 transcription and secretion from mitogen-activated normal T cells (49, 53). We have previously shown that EC can stimulate allogenic T cell proliferation (54) and do so because they provide costimulatory signals that result in augmented IL-2 transcription and secretion (34). EC signal T cells through LFA-3-CD2 and CD59-CD2 interaction and through a second, unidentified pathway (55). In this study, CD2 was found to be critical in mediating signals that resulted in enhanced IL-2 transcription and secretion when either B cells or EC were used as costimulators. In contrast, the CD28 pathway proved to be critical for B cell costimulation of transcription and IL-2 secretion, but was not productively engaged by EC. Thus, costimulation by two different accessory cells can be distinguished by the surface ligands through which they signal T cells. It is likely that both B cells and EC also signal through other ligands, not addressed in this report. An interesting finding was that transcription induced by B cells or EC was usually of comparable magnitude, whereas secreted IL-2 was usually greater from T cells costimulated by B cells. One explanation for this finding is that B cells do increase transcription beyond that registered by the transfected promoter, but do so through a site outside of the 600 base pairs we used. It is more likely that B cells, probably acting through CD28, are affecting IL-2 levels post-transcriptionally. Indeed, the increased secretion of IL-2 in response to B cell costimulation can be almost completely blocked by CTLA-4-Ig (Fig. 4). It has been reported that CD28 signaling has effects on IL-2 stability as well as on processing of nuclear transcripts and export from the nucleus (19, 20). A similar mechanism has also been described for regulation of IL-2 transcripts in the absence of CD28 signaling (56). These effects are likely specific for IL-2 mRNA and presumably would not affect the reporter gene.

A major conclusion of this study is that although different accessory cells costimulate T cells through different surface ligands, the same sites in the IL-2 promoter appear to be targeted. Thus, for both B cell and EC costimulation, the NF-κB and PAP-1 sites and, to a lesser extent, the NFAT, NFAP-1, and POC T sites are critical. This same combination of sites was also important for signaling by pairs of anti-CD2 mAbs. A surprising finding in these studies was the failure of the CD28RE mutation to reduce transcription induced by B cell costimulation, given that the mutation muted the effects of anti-CD28 mAb. T cell CD28 was productively engaged by B cell CD28 ligands in these experiments as CTLA-4-Ig fusion protein blocked augmentation of secreted IL-2. The most likely interpretation is that natural ligands for CD28 may deliver signals that differ from those produced by some anti-CD28 mAbs and that these signals do not induce activation of factors that bind to the CD28RE. Indeed, a recent report suggests that not all signaling through CD28 is identical. Using a panel of anti-CD28 mAbs, Nunes et al. (57) found that whereas a given pair of anti-CD28 mAbs may induce similar calcium fluxes in T cells, they can induce very different levels of IL-2 secretion. The mAbs recognize several different epitopes on CD28, and the implication is that signaling through CD28 (perhaps involving different epitopes) activates more than one signaling pathway. Alternatively, B cells (and potentially EC), unlike single mAbs, provide multiple costimulatory signals that result in expression of functionally redundant sets of transcription factors, obviating the role of factors bound to the CD28RE. This interpretation presupposes that CD28-mediated signals target other sites in addition to the CD28RE. Interestingly, the CD28RE has recently been shown to bind members of the NF-κB family of proteins, specifically NFκB1 (50), RelA (p65) and c-Rel (38), suggesting that CD28 may also activate the NF-κB site. However, in normal T cells, the NF-κB site and the CD28RE do not appear to be a functionally redundant pair since mutation of the NF-κB site cannot be compensated for by a functional CD28RE.

Although the same combination of IL-2 promoter elements is targeted by different costimulatory molecules, we have not ruled out the possibility that different combinations of nuclear factors may bind to these sites. Indeed, we have previously shown that when T cells are costimulated by EC, they express enhanced levels of c-Fos mRNA, and the AP-1 complex that binds to the PAP-1 site contains severalfold higher levels of Fos mRNA compared with cells activated in the absence of EC (34). Interestingly, a c-Fos knockout mouse has recently been generated that displays normal IL-2 induction, suggesting that other Fos proteins can substitute for c-Fos in these mice (58). There are also multiple NFAT proteins that may have differing roles under different conditions. Thus, it is possible that in response to different accessory cells, sequential expression of nuclear factors, expression of different family members, or expression of factors with longer half-lives, for example, may affect transcription. Indeed, IL-2 mRNA levels remain elevated longer in the presence than in the absence of EC costimula-
tion. The data presented in this study define the sites most relevant for future study of nuclear factor binding.

In conclusion, our findings suggest that the IL-2 promoter in normal T cells integrates signals by using a combination of several sites to activate transcription. This model is in agreement with a previous proposal describing fine tuning of IL-2 transcription (33). Furthermore, these studies emphasize the feasibility of working with normal nontransformed cells and the possibility that such cells may differ in significant ways from the tumor cells that have become "standard" models for the study of transcriptional regulation.

Acknowledgments—We thank Louise Benson and Gwen Davis for help with cell culture, Tom Taylor for help with FACS, Chris Benjamin for CD2 antibodies, Tim Springer for antibodies, and Gary Gray for CD28 antibodies and fusion proteins.

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