



In Vitro Transcription Faithfully Reflecting T-cell Activation Requirements*

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T-cell activation is a complex process mediated by cell membrane molecules including the T-cell antigen receptor (TCR), adhesive molecules, and cytokine receptors that collectively produce an increase in intracellular Ca**+, and activation of protein kinase C that initiate a genetic program resulting in immunologic function and irreversible differentiation. To understand how these cell membrane events are translated into a genetic regulatory cascade resulting in T-cell function, we have developed an in vitro transcription system, derived from Jurkat T-cells, which demonstrates inducible, cell-type-specific transcription following T-cell stimulation. Nuclear extracts from cells stimulated with phorbol 12-myristate 13-acetate and ionomycin, which activate protein kinase C and mimic physiological activation through the T-cell antigen receptor, transcribe an interleukin-2 (IL-2) enhancer (-326 to +24) template 5-fold more efficient than nuclear extracts from resting T-cells and 200-fold more efficient than extracts from Jurkat cells treated with phorbol 12-myristate 13-acetate or ionomycin alone. Further results demonstrate that in vitro transcription of the IL-2 enhancer is T-cell specific since nuclear extracts from rat liver and stimulated HeLa cells are unable to induce IL-2 transcription. The activation-dependent, T-cell-specific in vitro transcription system described herein would facilitate the dissection of signals that emanate from the T-cell surface resulting in IL-2 transcription and T-cell activation.

Occupation of the T-cell receptor (TCR) by antigenic peptides associated with major histocompatibility complex molecules in the presence of cytokines initiates a cascade of events in resting T-cells resulting in differentiation, proliferation, and immunological function. More than 100 genes are expressed during this 10-14-day process referred to as T-cell activation (1). The term activation has been used for this process since the metabolic activity and rates of protein synthesis of T-cells increase by several hundred-fold after effective antigen presentation. Many of the genes expressed during this orderly pathway, for example cytokines, function in coordinating cell fate decisions and proliferation of precursors of B cells, macrophages, and other cell types that are involved in the immune response (2, 3). Following this sequence of events, the cells revert back to their original metabolically resting state but are marked by the acquisition of immunologic memory and the expression of a new set of genes for at least several decades (4).

Molecules involved in initiating this process include the antigen receptor, adhesive molecules that probably play the function of nonspecifically holding the T-cell in proximity with the antigen presenting cell for the 30-60 min commitment period (5-7), and finally cytokine receptors such as those for IL-1 and IL-6. The collective activation of these cell surface molecules results in the production of a wide variety of second messengers including inositol phosphates (8), diacylglycerol (9), increase in intracellular calcium (10, 11), changes in cyclic nucleotide concentration (12), and activation of protein kinase C (13, 14). The activation of tyrosine kinases (pp60**+) and phosphatases also play an essential role in T-cell activation (15-19). In addition, accessory signals such as those provided by CD2, CD4, CD8 (20-22), or CD28 (23) may also be essential for full T-cell activation. These activation events are required for the transmission of signals emanating from the TCR-CD3 complex to genes in the nucleus where they are then integrated at the level of the promoter resulting in activation-specific transcription. In the laboratory, these physiologic signals can be mimicked by calcium ionophores (ionomycin) which elevate intracellular levels of calcium similar to activation through the TCR-CD3 complex (11, 24, 25), and tumor-promoting agents like phorbol 12-myristate 13-acetate (PMA) that activate protein kinase C (26).

As a model to study the signals required for activation-dependent transcription in T-cells, we focused on human interleukin 2 (IL-2) gene expression (1). IL-2 expression provides an attractive model for studying T-cell signal transduction since it is under stringent transcriptional control by the antigen receptor and, like T-cell activation, requires both elevated intracellular levels of calcium and activation of protein kinase C for expression (27, 28) it is essential for T-cell proliferation and immune function (28, 29), and 3) it is expressed at the time that T-cells become committed to activation. Thus, we speculate that the events required for the commitment of a T-cell to differentiate will be reflected in the regulatory regions of the IL-2 gene.

The IL-2 enhancer which is contained between -326 bp and +52 bp has been extensively studied and genetically dissected (30-36). From these studies, five functionally important sequences have been defined. Further analysis, using multimerized binding sites linked to an unrelated basal level promoter directing expression of chloramphenicol acetyltransferase reporter gene, indicates that two sites, A (-93 to

* This work was supported by National Institutes of Health Grant CA39612 (to G. R. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

‡ Supported in part by Immunology Postdoctoral Fellowship AI07290 and an American Cancer Society postdoctoral fellowship.

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The abbreviations used are: TCR, T-cell antigen receptor; PMA, phorbol 12-myristate 13-acetate; IL-2, interleukin-2; AdMLP, adenovirus major late promoter; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; bp, base pair; ARRE, antigen receptor response elements; Oct-1, octamer factor-1; OAP**, octamer-associated protein; NF-AT, nuclear factor of activated T-cells.

Vol. 267, No. 1, Issue of January 5, pp. 399-406, 1992
Printed in U.S.A.
and E (−285 to −255), respond to signals from the T-cell receptor (32). In contrast, accessory signals, mimicked by PMA stimulation fail to induce transcription of these constructs. Thus, these sequences are referred to as antigen receptor response elements or ARRE-1 and ARRE-2. ARRE-1 (−93 to −63) and ARRE-2 (−285 to −255) bind distinct nuclear proteins (32). ARRE-1 binds the ubiquitous POU protein octamer factor 1 (Oct-1) and a newly induced protein called octamer-associated protein (OAP) which functionally resembles the herpes simplex virus immediate-early transactivator VP16 (37). ARRE-2 binds a protein complex called nuclear factor of activated T-cells or NF-AT (34). Like the Oct-OAP complex, the appearance of the NF-AT complex is essential for IL-2 gene transcription (34). NF-AT binding activity, which requires RNA and protein synthesis, precedes the activation of IL-2 expression by 10–25 min suggesting that NF-AT is critical for the stringent control of IL-2 transcription and is one of the first genes induced in T-cell signal transduction.

Despite our basic understanding of the proteins which bind the ARREs, we know little about the biochemical events which activate these proteins to initiate transcription of the IL-2 gene. One powerful approach to understand and dissect the molecular mechanisms by which the antigen receptor communicates with the nucleus and activates early gene expression of T-cells is to reconstitute the in vitro events in a cell-free system. Thus, we have developed an activation-dependent, T-cell-specific in vitro transcription system. Our system, unlike any previously developed T-cell transcription system (38, 39), mimics the requirements for IL-2 expression and more generally for T-cell activation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Stimulation Conditions—**Jurkat cells were grown in RPMI 1640 with L-glutamine, 8% fetal calf serum (Irvine Scientific), with penicillin (100 units/ml) and streptomycin sulphate (100 μg/ml) at 5% CO₂ concentrations. Cells were split 1:3 36 h before stimulation. The morning of the stimulation, the Jurkat cells (1 × 10⁶ cells/ml) were centrifuged at 3500 rpm (2000 × g), in a GS-3 rotor for 10 min and then resuspended in fresh media to a concentration of 2 × 10⁶ cells/ml. In general, 2 μM ionomycin (Calbiochem) and 20 mg/ml PMA (Sigma) were used to stimulate the cells. During the 2-h stimulation, the cells were constantly shaking to prevent the yelling of cells on the bottom of the flask.

Hela S3 cells were grown in suspension minimum essential media ( Gibco) with 8% fetal calf serum (Irvine Scientific), with penicillin (100 units/ml) and streptomycin sulphate (100 μg/ml) and 2 mM L-glutamine. Hela S3 were stimulated for 2 h with 20 ng/ml PMA and 2 μM ionomycin.

**Plasmid Construction—**The IL-2 G-less plasmid was constructed by fusing the IL-2 enhancer (−326 to +24) to a 377-bp G-less cassette generously provided by R. Roeder (40) using polymerase chain reaction overlap extension techniques (41, 42). The IL-2 enhancer G-less cassette contained on a XhoI-BamHI fragment was inserted into a polylinker VP16 (37). ARRE-2 binds a protein complex called nuclear factor of activated T-cells or NF-AT (34). Like the Oct-OAP complex, the appearance of the NF-AT complex is essential for IL-2 gene transcription (34). NF-AT binding activity, which requires RNA and protein synthesis, precedes the activation of IL-2 expression by 10–25 min suggesting that NF-AT is critical for the stringent control of IL-2 transcription and is one of the first genes induced in T-cell signal transduction.

The HNF-1 (hepatocyte nuclear factor 1) G-less plasmid was constructed by inserting tandemly linked HNF-1-binding sites from rat β-fibrinogen promoter (−77 to −65) (44) into Xho-Sal polylinker sites in γ-fibrinogen G-less construct. The adenovirus major late promoter (AdMLP) G-less construct was a generous gift of Drs. M. Goralski and R. Roeder (Rockefeller University). Total size of the AdMLP G-less transcript is 280 nt.

**Preparation of Nuclear Extracts—**Jurkat and liver in vitro transcription nuclear extracts were essentially made as described by Gorski et al. (45, 46) with some exceptions. First, the cells were broken in 1.25 M sucrose-glycyl solution to reduce the amount of frictional heat generated during cell lysis. Second, 0.5% (v/v) nonfat dry milk was added to the homogenization buffer as had been previously described (46). Third, the Jurkat nuclei were fractionated on only one 2.0 M sucrose pad preceding salt extraction. Briefly, all manipulations were performed in the cold, and all solutions, tubes, and centrifuges were chilled to 4°C. Protease inhibitors, antasin (1 μg/ml), leupeptin (1 μg/ml), 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM benzamide were added to all buffers except the dialysis buffer. One mM dithiothreitol was added to all buffers. Following stimulation in the case for Jurkats, the cells (10⁸) were centrifuged in a GS-3 rotor, 3500 rpm (2,000 × g), for 10 min. The media was poured off, and the cells were resuspended with 40 ml of phosphate-buffered saline. Resuspended pellets were then centrifuged 1,000 rpm (200 × g), 10 min in a Beckman GPR tabletop centrifuge. The cell pellet was resuspended in 10 ml of homogenization buffer (10 mM Heps, pH 7.6, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1.25 mM sucrose, 10% glycerol (v/v), 0.6% nonfat dry milk (v/v)). An aqueous 0.1 g/ml nonfat dry milk solution was centrifuged for 10 min in a SS-34 rotor at 10,000 rpm (11,950 × g) to remove undissolved milk solids before adding to any solution.

The cells were then dissolved (yellow gel homogenizer) until broken using a 1.5 horsepower drill press (Jet Tools Inc.) at high speed. Cells were checked for lysis. Generally, greater than 80% of the cells were lysed. Following lysis, 46 ml of 2 mM sucrose homogenization buffer (10 mM Heps, pH 7.6, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 mM sucrose, 10% glycerol (v/v), 0.6% nonfat dry milk (v/v)) were added to the beads to be dialyzed cells to be homogenized (28 ml) were layered on to 10 ml sucrose pads (10 mM Heps, pH 7.6, 25 mM KCI, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 mM sucrose, 10% glycerol (v/v)) and centrifuged at 24,000 rpm for 60 min in a SW 28 rotor (100,000 × g) to pellet the chromatin.

In preparing centrifugation, the tubes were quickly removed and the supernatant transferred to another tube before the pellet for centrifugation. The supernatant was centrifuged at 25,000 rpm, 40 min in a Ti 50 rotor (150,000 × g) to pellet the chromatin.

In preparing centrifugation, the tubes were quickly removed and the supernatant transferred to another tube before the pellet for centrifugation. The supernatant was centrifuged at 25,000 rpm, 40 min in a Ti 50 rotor (150,000 × g). At this point, the pellet was immediately resuspended in dialysis buffer (25 mM Heps, pH 7.6, 40 mM KCI, 0.1 mM EDTA, 10% glycerol (v/v)). Protein extracts from 1 × 10⁶ Jurkat cells were resuspended in 200–300 μl of dialysis buffer resulting in a final protein concentration of 10 mg/ml. Extracts were dialyzed twice for 2 h in the cold against 100 volumes of dialysis buffer. During dialysis a precipitate forms that at the end of dialysis was removed by centrifugation in a microfuge (Brinkman Instruments) at a setting of 14 for 7 min. Protein concentration was determined with a Bio-Rad protein assay kit using bovine serum albumin as a standard. Protein extracts were frozen in small aliquots on dry ice and immediately stored in liquid nitrogen.

**HeLa S3 nuclear extracts were made as previously described (47).** Sedimentation density gradient centrifugation (5% and 20%) contained 22 μg/ml of circular DNA template (3000 ng of the test construct, 40 ng of the AdMLP G-less construct, and 360 ng of poly(cytidylate) (Pharmacia LKB Biotechnology Inc.) and between 3–5 mg/ml nuclear protein extract in a buffer containing 25 mM Heps, pH 7.6, 25 mM KCI, 1 mM dithiothreitol, 0.6% nonfat dry milk, 0.5 M EDTA, 7 μg of [α-32P]UTP (Amersham Corp., 3000 Ci/mmol), 0.5 mM 5′-G-methyl GTP (Pharmacia), 150 units of RNase T1 (Bethesda Research Laboratories), 12 units of RNase inhibitor (Amersham Corp.), and 12% glycerol (v/v). EDTA and dithiothreitol were con-
Cell-type-specific, Activation-dependent in Vitro Transcription—By using nuclear extracts from Jurkat cells, a human T-cell leukemia-derived cell line, in vitro transcription was examined using the IL-2 enhancer, as well as multimerized ARRE-2- and ARRE-1-binding sites linked to a 377-bp G-less cassette obtained from p(CAT)$_2$ (40) as illustrated in Fig. 1A. As an internal control for transcription efficiency and correction of handling errors, we have used the adenovirus major late promoter linked to a truncated 280-bp G-less cassette (AdMLP G-less) as represented schematically in Fig. 1B. The adenovirus major late promoter is a strong promoter and effectively initiates transcription in most in vitro systems. Fig. 1C illustrates in vitro transcription results from nuclear extracts of stimulated and resting Jurkat cells using the IL-2 G-less template. Immediately apparent is the efficient transcription (fold induction of 5.6) of the IL-2 G-less template using nuclear extracts from stimulated cells as compared to nuclear extracts from nonstimulated cells (Fig. 1C, compare lanes 1 and 2). Furthermore, transcription is abolished in the presence of 1 µg/ml α-amanitin (Fig. 1C, lanes 3 and 4) indicating that expression is dependent on DNA polymerase II.

To optimize conditions for our in vitro transcription system, the effects of extract concentration on fold induction of the IL-2 G-less construct were examined as shown in Fig. 2. The total amount of DNA (800 ng) per reaction as well as volume of the reaction was kept constant, and the extract concentration was increased from 1.25 to 7.5 µg/ml. Transcription from the IL-2 G-less and AdMLP G-less appeared throughout the range of concentrations; however, optimal transcription of the promoter constructs was observed between approximately 2.5 and 6.25 µg/ml with the greatest transcriptional fold induction between nonstimulated and stimulated nuclear extracts witnessed at 3.75 µg/ml. As seen in other in vitro transcription systems, the ratio of DNA to protein in the reaction is critical for efficient transcription (45, 48, 49). However, it is important to note that under all extract concentrations the IL-2 G-less construct was transcribed more efficiently in the stimulated Jurkat nuclear extracts than the nonstimulated nuclear extracts.

In Vitro Transcription of the IL-2 Enhancer Is Dependent on Two Distinct Signals—The net effect of triggering the constellation of cell membrane molecules involved in T-cell activation can be largely mimicked by agents that activate protein kinase C (PMA) and increase intracellular Ca$^{2+}$ (ionomycin). Curiously, activation of the antigen receptor stimulates phosphatidylinositol 4,5-bisphosphate hydrolysis and, thus, the production of inositol 1,4,5-trisphosphate and diacylglycerol which induce the release of Ca$^{2+}$ from intracellular stores and activate protein kinase C, respectively (11, 50); yet, these signals are not sufficient to activate IL-2 transcription implying that accessory signal(s) are required. Cytokines such as IL-1 or IL-6 provide the second stimulus for T-cell activation and can be mimicked by agents that activate protein kinase C (14). However, IL-1 and IL-6 do not physiologically activate protein kinase C (51), suggesting that protein kinase C is not the physiologic second signal that tumor promoters mimic. Despite these complexities, we sought to determine if in vitro transcription, like T-cell activation, requires the concordant delivery of these two signals for inducible IL-2 transcription.

Results shown in Fig. 3 demonstrate that fully activated in vitro transcription of the IL-2 enhancer is dependent on both elevated intracellular calcium and activation of protein kinase C (Fig. 3, compare lanes 1-5). Although low-level transcription occurs from the IL-2 G-less template in nuclear extracts made from Jurkat cells stimulated with PMA or ionomycin alone (Fig. 3, lanes 2 and 3), only when both distinct signals are provided do we observe high levels of in vitro transcription (Fig. 3, lanes 4 and 5). The differences observed in activated transcription of the IL-2 G-less between extracts stimulated with the monoclonal antibody C305 and PMA versus PMA and ionomycin reflect the reduced ability of soluble antibodies to the T-cell receptor to generate inositol phosphates and translocate protein kinase C (52, 53). However, it is clear that fully activated in vitro transcription of the IL-2 G-less demands the same stringent requirements as in vivo IL-2 expression.

To further test the fidelity of our activation-dependent in vitro transcription system, we tested ARRE-1 G-less and ARRE-2 G-less templates. Previous studies using transient expression assays identified ARRE-2- and ARRE-1-binding sites as targets for signals emanating from the T-cell receptor (32). Similarly, in vitro transcription results show that multimerized ARRE-1- and ARRE-2-binding sites activate transcription of the γ-fibrinogen minimal promoter in response to ionomycin which is believed to mimic physiological stimulation through the antigen receptor (24) (Fig. 3, lanes 8 and 13). However, like IL-2 G-less transcription, maximum transcription of ARRE-1 and ARRE-2 G-less templates requires that cells be stimulated with both PMA and ionomycin. These results further demonstrate that our in vitro transcription system accurately reflects precisely the same complex requirements as physiologic expression of the IL-2 gene.

Evidence That NF-AT Is Required for Activated IL-2 Transcription—To demonstrate that sequences within the IL-2 enhancer are essential for activated in vitro transcription, we constructed an internal deletion mutant (ID 273/285) of the IL-2 enhancer, which disrupts the ARRE-2-binding site. Previous results using transient expression assays demonstrated that this internal deletion mutant reduces transcription to 30% of wild type IL-2 transcription (32). Surprisingly, when tested in our in vitro transcription system, this construct was as efficiently transcribed as the wild-type IL-2 G-less construct (Fig. 4A, lanes 1-4). These results imply that binding of NF-AT to its cognate site (ARRE-2) is not required for in vitro transcription of the IL-2 enhancer. Alternatively, NF-AT may activate transcription from additional sites within the IL-2 enhancer.

To distinguish between these interpretations, we titrated NF-AT binding activity using an ARRE-2-specific oligonucle-
A.

**Diagram of the IL-2 enhancer and IL-2 enhancer-derived G-less constructs.** A, map of the IL-2 enhancer (-226 to +24 bp of IL-2) fused to the G-less cassette. Previously identified DNase I protected regions are noted by boxes along with the identification of the sites (A-E) and the name(s) of proteins which complex with these sites. Transcription of the IL-2 G-less construct generates a 401-nt transcript. The ARRE-1 G-less construct contains four Oct-1/OAP-binding sites (-93 to -66 bp of IL-2) linked to an unrelated minimal promoter, γ-fibrinogen (-52 to +1 bp), that is ligated to the G-less construct. The ARRE-2 G-less template has three NF-AT-binding sites (-286 to -257 bp of IL-2) linked to the γ-fibrinogen promoter (-52 to +1 bp) and attached to the G-less cassette. Transcription of both of these constructs generates a 383-nt transcript. B, map of the AdMLP G-less construct. Transcription of the AdMLP G-less template generates a 280-nucleotide transcript. C, in vitro transcription of the IL-2 G-less template using nuclear extracts from nonstimulated (lanes 1 and 3) and stimulated (lanes 2 and 4) Jurkat cells in the absence (lanes 1–2) or presence (lanes 3–4) of α-amanitin. The Jurkat cells were stimulated for 2 h with 20 ng/ml PMA and 2 μM ionomycin. In vitro transcription of the AdMLP G-less construct (lower transcript, lanes 1 and 2) provides an internal control for handling errors and transcription efficiency among different nuclear extracts. Normalized fold induction was determined as indicated in experimental procedures.

B.

Contrast to transient expression assays, since the functional contribution of a putative transcription factor can be rapidly assessed by specifically depleting it.

**Jurkat in Vitro Transcription Extracts Show Promoter Specificity**—To demonstrate that the transcriptional induction observed in PMA and ionomycin stimulated Jurkat nuclear extracts is promoter specific, we tested a G-less cassette construct containing two tandemly linked binding sites for a HNF-1 linked to γ-fibrinogen promoter and compared its transcription to that of the IL-2 G-less construct. The HNF G-less construct should be relatively silent in T-cell extracts since HNF-1 is not present in Jurkat nuclear extracts (54). As shown in Fig. 5A, HNF G-less shows basal level transcription in both nonstimulated and stimulated nuclear extracts (lanes 1 and 2) but fails to demonstrate inducible activation-dependent transcription using nuclear extracts from stimulated Jurkat cells. Similarly, the AdMLP G-less internal control is equally transcribed in nuclear extracts from non-

otide and tested the transcriptional competence of the nuclear extract. As shown in Fig. 4B, depletion of NF-AT reduced transcription of the IL-2 G-less cassette to 24% of wild type induction (Fig. 4B, compare lanes 2 and 6). Transcription of the AdMLP G-less construct was unaffected indicating that only NF-AT was specifically depleted. To further confirm that the reduction in IL-2 G-less transcription was due to specific depletion of NF-AT by ARRE-2, we carried out similar experiments using the pUC 18 polylinker (EcoRI-HindIII) as a nonspecific competitor and observed only 15% reduction in IL-2 G-less transcription at 100-fold molar excess of the pUC polylinker (data not shown) far less than the 76% reduction in IL-2 G-less transcription demonstrated using the ARRE-2 oligonucleotide as a competitor. These results demonstrate that NF-AT is essential for activated in vitro transcription of the IL-2 enhancer and suggests that NF-AT binds to more than one site within the IL-2 enhancer. These data highlight the utility of our in vitro transcription system, in contrast to transient expression assays, since the functional contribution of a putative transcription factor can be rapidly assessed by specifically depleting it.
plates are poorly transcribed. Therefore, plus PMA (20 ng/ml) stimulated plate contains high affinity binding sites for general transcription using stimulated nuclear extracts from nonstimulated (ns) and stimulated (s) Jurkat cells are indicated above each pair of lanes.

![Fig. 2. Effects of extract concentration on in vitro transcription of the IL-2 G-less construct.](image)

**Fig. 2.** Effects of extract concentration on in vitro transcription of the IL-2 G-less construct. The concentration of nuclear extracts used and the corresponding transcriptional fold induction observed between nuclear extracts from nonstimulated (ns) and stimulated (s) Jurkat cells are indicated above each pair of lanes.

stimulated as in stimulated nuclear extracts (Fig. 5A, lower band, lanes 1–4). In contrast, IL-2 G-less template demonstrates induced transcription using stimulated nuclear extracts as compared to nonstimulated nuclear extracts (Fig. 5A, lanes 3 and 4). These results indicate that activated transcription of the IL-2 G-less in nuclear extracts from PMA/ionomycin-stimulated Jurkat cell is due to the positive influences of newly synthesized or activated T-cell-specific factors and is not due to a general transcriptional promiscuity of the stimulated extracts.

Alternatively, it could be argued that the IL-2 G-less template contains high affinity binding sites for general transcription factors unlike the HNF G-less and AdMLP G-less constructs which results in induced transcription. To dismiss this hypothesis, we tested several G-less constructs using nuclear extracts from rat liver. As shown in Fig. 5B, lane 2, the HNF G-less construct which contains binding sites for a liver-enriched transcription factor (HNF-1) (44) is readily transcribed in liver nuclear extracts where as the γ-fibrinogen minimal promoter, ARRE-1, ARRE-2, and IL-2 G-less templates are poorly transcribed. Therefore, in vitro transcription of the IL-2-derived G-less constructs require T-cell-specific factors.

Finally, to be confident that the increased transcription we observe using nuclear extracts from stimulated Jurkats is not due to nonspecific effects of PMA/ionomycin treatment, we tested transcription from PMA/ionomycin-stimulated HeLa cells. HeLa cells were stimulated with PMA and ionomycin, nuclear extracts were prepared (47), and transcription was examined. Extracts from PMA/ionomycin-stimulated HeLa cells failed to show activated transcription of the G-less constructs tested (Fig. 6, lanes 1–10) even though the AdMLP G-less construct was actively transcribed in these same extracts (Fig. 6, lower transcript). We are confident that the HeLa cells were stimulated since we observed the appearance of a NF-κB complex using a labeled immunoglobulin G NF-kB-binding site in a gel mobility shift assay (data not shown). Therefore, the data further suggest that induced transcription of the IL-2 enhancer-derived G-less constructs requires T-cell-specific factors that are provided only in nuclear extracts from activated Jurkat T-cells.

**Fig. 3.** In vitro transcription of the IL-2 enhancer is dependent on two distinct signals. Comparison of IL-2 transcription from nuclear extracts stimulated under different conditions. Non-stimulated (lane 1); PMA (20 ng/ml) stimulated (lane 2); ionomycin (2 μM) stimulated (lane 3); C205 (1:10,000 dilution of acites fluid) plus PMA (20 ng/ml) stimulated (lane 4); PMA (20 ng/ml) and ionomycin (2 μM) stimulated (lane 5). Transcription of the ARRE-1 G-less template (lanes 6–10) and ARRE-2 G-less template (lanes 11–15) using the same set of nuclear extracts as described above. The Jurkat cells were stimulated for 2 h. Normalized fold induction was determined as described under “Experimental Procedures.”

**DISCUSSION**

In this report, we have established an activation-dependent, T-cell-specific in vitro transcription system which accurately reflects the complex requirements for IL-2 transcription and more generally T-cell activation. In nuclear extracts derived from PMA- and ionomycin-stimulated Jurkat T-cells, the IL-2 enhancer is 5-fold more transcriptionally active than in nuclear extracts from resting cells. The activated expression of the IL-2 enhancer in stimulated nuclear extracts is specific and not due to a general transcriptional promiscuity of the stimulated extracts since these same extracts fail to inducibly transcribe a promoter template containing multimerized binding sites for a transcription factor which is absent in T-cell extracts. In fact, all experiments of the IL-2 enhancer demands the positive influences of T-cell-enriched transcription factors. Oligonucleotide titration of transcription factors, as well as, deletions of DNA regulatory regions drastically reduce in vitro IL-2 transcription. These data indicate that our in vitro transcription system mimics physiologic in vivo IL-2 gene expression.

The utility of a cell-type-specific transcription system is that specific effects of putative transcription factors can be directly tested (55–62). For instance, purified, in vitro translated, bacterially expressed, or mutated proteins can be added to nuclear extracts from resting or stimulated Jurkat cells and analyzed for their ability to potentiate or repress transcription of a specific promoter. This is in contrast to the transfection of DNAs encoding putative transcription factors that may indirectly affect transcription by activating intermediate steps in a signal transduction pathway. The ability to carry out such add-back experiments, highlights the power of in vitro transcription systems to facilitate the identification and characterization of factors essential to regulate gene expression.

**Cell-type-specific in Vitro Transcription**—Two lines of evidence suggest that in vitro transcription of the IL-2 enhancer, like in vivo expression, is controlled by the induction of positive trans-acting factors. First, inducible IL-2 transcription is observed following T-cell stimulation. Indeed, incubation of templates with nonstimulated nuclear extracts for 15 min on ice followed by the addition of stimulated nuclear extracts results in activated transcription suggesting that transcription requires positive trans-acting factors not derepression of inhibitory factors. However, under these cir-

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2 M. Flanagan and G. Crabtree, unpublished observations.
A. G-less constructs

B. molar excess ARRE-2 oligo

Fig. 4. Evidence that NF-AT is required for activated in vitro transcription of the IL-2 enhancer.

A. A, analysis of an ARRE-2 deletion mutant. In vitro transcription of the wild type IL-2 enhancer (lanes 1 and 2) and an internal deletion mutant ID 279/263 (lanes 3 and 4) using nuclear extracts from nonstimulated (lanes 1 and 3) and stimulated (lanes 2 and 4) was analyzed and compared. Fold induction was determined for both IL-2 G-less and ID 279/263 G-less transcription. Calculated fold induction from the wild type IL-2 G-less cassette was set to 100%. B, titration of NF-AT binding activity. The IL-2 G-less template was transcribed in nuclear extracts from nonstimulated (lanes 1, 3, and 5) and stimulated (lanes 2, 4, and 6) Jurkat cells in the presence of increasing molar excess of ARRE-2 oligonucleotide as indicated. Fold induction of the IL-2 transcription in the absence of ARRE-2 oligonucleotide was determined and set to 100%.

Fig. 5. Activated transcription in nuclear extracts from stimulated Jurkat cells demonstrates promoter specificity. The HNF-1 G-less construct, which contains tandemly linked binding sites for the HNF-1 linked to a minimal promoter (γ-fibrinogen, −52 to +1 bp) directing expression of a G-less cassette, shows basal level expression in nuclear extracts from both nonstimulated (lane 1) and stimulated (lane 2) Jurkat cells. The IL-2 G-less template demonstrates transcriptional inducibility (compare lanes 3 and 4) using the same nuclear extracts. B, in vitro transcription of the IL-2 G-less construct is dependent on T-cell-specific factors. Transcription of several G-less promoter constructs was analyzed using nuclear extracts from rat liver. The γ-fibrinogen minimal promoter (lane 1), ARRE-1 G-less (lanes 3), ARRE-2 G-less (lanes 4), and IL-2 G-less (lane 5) are weakly transcribed. In contrast, the HNF-1 G-less (lane 2) is efficiently transcribed.

cumstances, we may fail to observe the effects of a transcriptional repressor. Since relatively few DNA templates form active transcriptional complexes in vitro, approximately 1-2% (45, 63), the excess templates may functionally titrate repressor molecules while leaving a substantial number of templates available for transcription. One report by Nabel et al. (64) suggested that the IL-2 enhancer is negatively regulated by a repressor bound to the ARRE-1 site; however, we and others have been unable to confirm these results (65). Accordingly, they have recently published data that concur with our original analysis (32) that indicate a positive factor Oct-1 functions at this site (65).

Second, deletions within the IL-2 enhancer and depletion of T-cell-specific transcription factors, like NF-AT, result in the loss of transcriptional activity. Based on previous results using transient expression assays, at least five functionally important sequences located between −326 and −51 bp of the IL-2 enhancer have been defined. Removal of sequences encompassed by either ARRE-2 or ARRE-1 results in dramatic loss of transcriptional activity (32). Parallel experiments utilizing our in vitro transcription system also demonstrate, at least qualitatively, that deletion of or point mutations within ARRE-1 reduces IL-2 transcription by disrupting Oct-1 and OAPβ, an inducible Oct-1-associated protein, binding (37).

In contrast, as shown in Fig. 4A, removal of sequences encompassed by ARRE-2 fails to demonstrate reduced in vitro transcription of an IL-2 enhancer template. Gel mobility shift assays, DNase I footprinting, and methylation interference data indicate that ARRE-2 is bound by the T-cell-enriched transcription factor NF-AT (32, 34). Based on the characteristics of NF-AT, namely its selective expression in activated T-cells (34), its induction just prior to IL-2 gene activation, its sensitivity to immunosuppressive drugs (66, 67), and its
implication in the cause of a recent reported case of severe combined immune deficiency syndrome (68), it follows that IL-2 expression is linked to the formation of NF-AT. Thus, the paradoxical nature of these data imply that NF-AT binds to additional sites within the IL-2 enhancer to activate transcription. Accordingly, titration of NF-AT from stimulated nuclear extracts drastically and specifically diminished transcription of the IL-2 reporter template. Close inspection of the nucleotide sequence of the IL-2 enhancer identified an additional putative NF-AT-binding site (−143 to −124 bp). Recent data suggest that sequences between -156 and -104 of the IL-2 enhancer bind a factor which appears to be NF-AT based on methylation interference results and sensitivity to immunosuppressive drugs (69, 70). Taken together these results may explain, given the apparent importance of NF-AT in IL-2 transcription, why original deletion mutations tested in transient expression assays still retained 30% of their wild type activity (32).

Further evidence that in vitro transcription of the IL-2 enhancer requires T-cell-specific factors and, therefore, demonstrates cell-type-specific transcription, results from the inability of HeLa and rat liver nuclear extracts to transcribe the IL-2 enhancer templates despite the ability of both extracts to efficiently transcribe the AdMLP control template. Again, we do not believe HeLa or liver extracts contain an inhibitor of transcription since in mixing experiments the in vitro transcription activity of stimulated Jurkat nuclear extracts is not repressed by either HeLa or liver nuclear extracts. Instead, the failure of these extracts to activate transcription results from the absence of T-cells-specific factors; however, this is not the entire story. Although HeLa and liver nuclear extracts do not contain NF-AT binding activity, both extracts contain Oct-1 (71, 72) and an OAP4' like protein, as distinguished by gel mobility shift assays. Yet, post-translational modifications and T-cell-specific coactivators may play a role in ARRE1-directed transcription in T-cells.

**Dissection of T-cell Signaling Pathways—**Many of the interleukins such as IL-2 and lymphotoxin are remarkably toxic molecules, thus, the process of T-cell activation must be kept under the stringent control of the antigen receptor so that occurring with less specific signals, such as circulating cytokines. As opposed to previously defined T-cell-derived in vitro transcription systems (38, 39), ours mimics the specific in vivo two-signal requirement for T-cell activation. This has allowed the demonstration that NF-AT and OAP4' are positively acting, tissue-specific transcription factors (37). In the future, this system should provide vital information for understanding the steps in a signal transmission cascade that transduces signals from the antigen receptor and its associated cell membrane accessory molecules to the nucleus.

**Acknowledgments—**We would like to thank members of the Crabtree lab for stimulating discussions. We would also like to thank Dirk Mendel for teaching us how to prepare in vitro transcription extracts, and Biaze Cortesley for critically reading the manuscript.

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