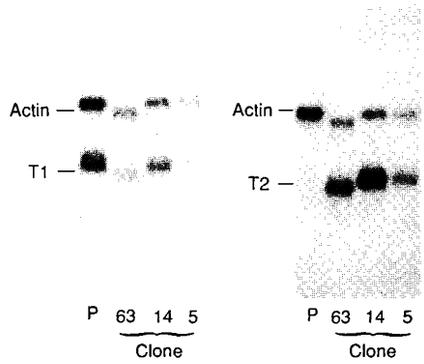


**Fig. 3.** Expression of T1 and T2 by cell clones. Polyadenylated RNA was separated by electrophoresis on a 2.2*M* formaldehyde denaturing gel and transferred to nitrocellulose (*30*). After baking and prehybridization, the filter was probed successively with $^{32}$P-labeled T2, T1, and actin and was washed at 80°C for 30 min in 0.1% SDS and 0.1% saline sodium citrate (SSC) between probes. RNA hybridizing with T1 was present in parental cells and in idiotype-negative clones, but T2 was expressed only in idiotype-negative clones. P, parental.

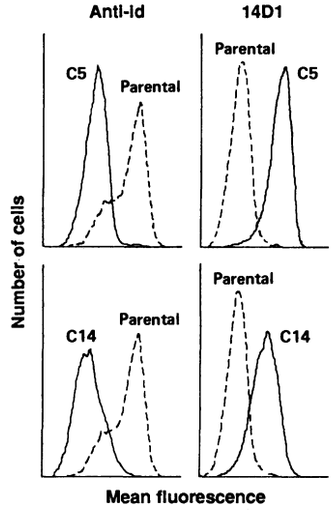


**Fig. 4.** Expression of Oz and idiotypic determinants by cell clones. Parental cells (dashed lines) and variants C5 and C14 (solid lines) were examined by indirect immunofluorescence with anti-idiotype (anti-id) and monoclonal antibody 14D1 (directed against $Oz^-$) and analyzed with the FACS 440 (Becton Dickinson). Fluorescence intensity is displayed on a log scale.

The two different idiotype-negative clones rearranged the same V-region gene. This implies that Vλ gene usage by the tumor variants was restricted. Preferential V gene usage has been described for the heavy chain locus in normal B cells during murine (*17*) and human (*18*) fetal development. In these cases the preferred heavy chain V-region genes ($V_H$ genes) were those proximal to the J region gene cluster. Mapping of the human Vλ genes may provide an explanation for the repetitive use of one particular Vλ gene by this tumor.


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# Regulation of Lymphokine Messenger RNA Stability by a Surface-Mediated T Cell Activation Pathway


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**Quiescent T cells can be induced to express many genes by mitogen or antigen stimulation. The messenger RNAs of some of these genes undergo relatively rapid degradation compared to messenger RNAs from constitutively expressed genes. A T cell activation pathway that specifically regulates the stability of messenger RNAs for the lymphokines interleukin-2, interferon-γ, tumor necrosis factor–α, and granulocyte-macrophage colony-stimulating factor is induced by stimulation of the CD28 surface molecule. This pathway does not directly affect the steady-state messenger RNA level, transcription, or messenger RNA half-life of other T cell activation genes, including c-*myc*, c-*fos*, IL-2 receptor, and the 4F2HC surface antigen. These data show that stimuli received at the cell surface can alter gene expression by inducing specific changes in messenger RNA degradation.**


ALTHOUGH MUCH HAS BEEN learned about the transcriptional regulation of gene expression (*1*), relatively little is known about how mRNA stability contributes to gene expression (*2*). In both prokaryotic and eukaryotic cells some mRNAs are relatively short-lived while other mRNAs are extremely stable (*2*). In bacteria, many of the genes that encode short-lived mRNAs are responsive to growth conditions (*3*), a short mRNA half-life possibly allowing for rapid modulation of the expression of cell growth–associ-


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ated genes in response to the environment. Similarly, in eukaryotic cells, many genes associated with cell growth, including the nuclear proto-oncogenes, genes associated with DNA replication, and several transcription factor genes, encode mRNAs with short half lives (*1, 4–6*). The mRNAs for several of these genes are stabilized during stimulation by serum of serum-deprived cells or treatment with cycloheximide, a protein synthesis inhibitor (*5, 6*). These data suggest that there may be a cellular mRNA degradation system that is labile and sensitive to the cellular environment (*5–7*).

Some eukaryotic genes that encode secretory molecules also have mRNAs with short half lives (*8*). These genes all encode highly regulated extracellular molecules such as peptide hormones, lymphokines, and cytokines. We now report a novel surface-mediated T cell activation pathway that specifically regulates the mRNA stability of a group of lymphokines sharing certain structural characteristics in their 3′ untranslated regions.

T cells are important regulators of in vivo immune responses, and the specificity of a T cell–initiated immune response is mediated by a complex of the T cell receptor and CD3 (TCR-CD3) (*9*). Interaction of this complex with antigen in association with a major histocompatibility complex protein can result in the induction of T cell effector functions such as lymphokine production and cytolytic activity (*10*). A number of additional T cell surface molecules appear to contribute to the control of T cell immune responses (*11*). One such molecule is CD28, a 44-kD glycoprotein that is a member of the immunoglobulin supergene family and is expressed as a homodimer on a major subset of human T cells (*12*). We have previously shown that when normal human T cells that have been activated by cross-linking of the TCR-CD3 complex are exposed to an antibody to CD28 (anti-CD28), there is a marked enhancement of the induction of interleukin-2 (IL-2) mRNA (*13*). The expression of three additional lymphokines that have been reported to be coordinately expressed in some murine T cell clones in conjunction with IL-2 (*14*) was therefore examined. These genes include tumor necrosis factor–α (TNF-α),

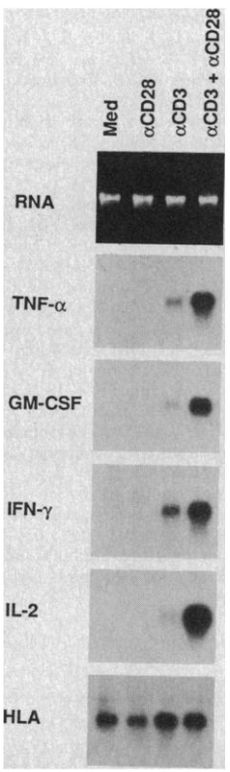


**Fig. 1.** Effects of CD28 stimulation on TNF-α, GM-CSF, IFN-γ, IL-2, and HLA class I mRNA expression. The CD28$^+$ T cells were purified (*13*) and cultured at 2 × 10$^6$ cells per milliliter in the presence of medium alone (Med), soluble monoclonal anti-CD28 9.3 (αCD28), monoclonal anti-CD3 G19-4 immobilized to plastic (αCD3), or anti-CD3 immobilized to plastic together with soluble anti-CD28 (αCD3 + αCD28). The anti-CD3 was immobilized by adsorbing the antibody to the surface of plastic tissue culture plates, which was necessary to achieve the cross-linking of the TCR-CD3 receptor required for proliferation of purified T cells (*18*). The amount of anti-CD3 used in each well was that which induced maximal proliferation of peripheral blood T cells at 72 hours (1 μg/ml of cells). Anti-CD28 was used at a dose of 1 μg/ml. The cells were harvested after 6 hours of culture, and total cellular RNA was isolated (*13*). RNA blots were prepared from equalized samples of RNA and hybridized sequentially with probes specific for TNF-α, GM-CSF, IFN-γ, IL-2, and HLA class I genes (*23*). The resulting autoradiograms are shown. The upper panel represents ethidium bromide staining of the 28*S* ribosomal RNA band from the equalized RNA samples used to prepare the RNA blots. The data presented are representative of five separate experiments. Separate experiments have demonstrated that anti-CD28 enhances anti-CD3–induced lymphokine gene expression in both the CD4$^+$ and CD8$^+$ subsets of CD28$^+$ T cells (*15*).

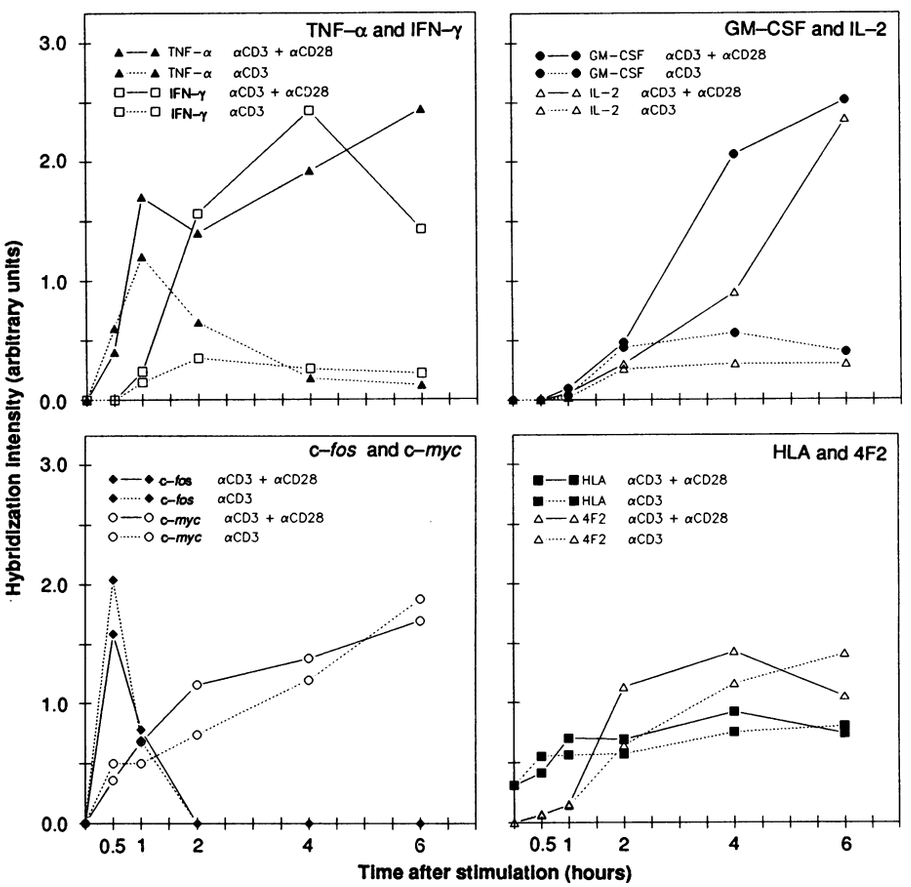


**Fig. 2.** Kinetics of augmentation by anti-CD28 of TNF-α, GM-CSF, IFN-γ, and IL-2 mRNA expression of normal human T cells stimulated with anti-CD3. CD28$^+$ T cells were cultured with anti-CD3 immobilized to plastic or anti-CD3 in the presence or absence of soluble anti-CD28. Cells were harvested at the times shown after stimulation, and total cellular RNA was isolated. RNA blots were prepared from equalized RNA samples and hybridized to specific probes for TNF-α, GM-CSF, IFN-γ, IL-2, c-*fos*, c-*myc*, 4F2HC (4F2), and HLA class I genes as described in the legend to Fig. 1. Hybridization intensity of the gene-specific probes was measured by scanning densitometry (*6*). The data presented are from a single experiment, but are representative of the results of five independent experiments.

granulocyte-macrophage colony-stimulating factor (GM-CSF), and interferon-γ (IFN-γ). All four lymphokines are associated with T cell regulation of delayed-type hypersensitivity responses.

Resting CD28$^+$ T cells do not express any of the four lymphokine genes studied (Fig. 1). Cross-linking of the TCR-CD3 complex with immobilized antibodies to CD3 (anti-CD3) induced the expression of all four genes. Although stimulation with anti-CD28 alone failed to induce expression of these genes, stimulation of CD28 was found to synergize with TCR-CD3 cross-linking to cause a 5- to 20-fold enhancement in the expression of all four lymphokines. This augmentation in lymphokine gene expression by anti-CD28 occurred at all doses of anti-CD3 tested over a 50-fold range (0.015 μg to 1.0 μg per milliliter). When cells were stimulated with 1.0 μg/ml, a concentration that maximized anti-CD3–induced T cell proliferation, only the expression of the four lymphokine genes was enhanced by anti-CD28 costimulation (Fig. 1). Under these conditions, anti-CD28 did not augment T cell proliferation or the expression of c-*myc*, c-*fos*, IL-2 receptor, and 4F2HC genes (*15*). Therefore, in all subsequent experiments we have used an anti-CD3 dose that has been titrated to induce maximal T cell proliferation in the absence of co-mitogens.

The kinetics of TNF-α, GM-CSF, IFN-γ, and IL-2 mRNA expression after stimulation of normal human T cells with anti-CD3 were markedly different (Fig. 2). Whereas TNF-α was found to be induced as an early response gene with peak expression at 1 hour, GM-CSF, IFN-γ, and IL-2 all required several hours of stimulation with anti-CD3 for the induction of peak expression. The induction of TNF-α by stimulation with anti-CD3 can occur in the absence of new protein synthesis, whereas the induction of GM-CSF, IFN-γ, and IL-2 expression in response to stimulation with anti-CD3 failed to occur in the presence of the protein synthesis inhibitor cycloheximide (*16*). Despite these differences, costimulation with anti-CD28 was capable of augmenting mRNA expression of each lymphokine at the indicated times after their induction by stimulation with anti-CD3. In separate experiments, maximal induction of all four lymphokines was found to occur between 4 and 8 hours after costimulation with anti-CD3 and anti-CD28. The expression of the T cell activation genes, c-*myc*, c-*fos*, and 4F2HC, and the constitutively expressed human leukocyte antigen (HLA) class I genes were also studied. Despite markedly different patterns of expression in response to stimulation with anti-CD3, costimulation with anti-CD28 had no significant effect on the steady-state mRNA levels of these genes.

We investigated the role of transcriptional regulation in the induction of lymphokine expression by anti-CD3 and anti-CD3 together with anti-CD28 by performing run-on transcription assays on CD28$^+$ peripheral blood T cells that had been cultured in the presence of medium alone, anti-CD3, or anti-CD3 together with anti-CD28 (Fig. 3). Under all three conditions, an equivalent level of transcription of HLA class I, glyceraldehyde-3-phosphate dehydrogenase, and 28*S* ribosomal RNA genes was found. The transcription of the 4F2HC gene in the cells cultured in the presence of medium alone is consistent with the reported regulation of 4F2 gene expression by transcriptional attenuation in quiescent cells (*17*). Treatment with anti-CD3 alone was capable of inducing the transcription of a variety of T cell activation genes, including IL-2, IFN-γ, GM-CSF, TNF-α, 4F2HC, and IL-2 receptor. The addition of an anti-CD28 stimulus had no effect on the transcription of any of these genes. Our data are consistent with the results of previous experiments showing that stimulation of resting T cells with anti-CD3 is sufficient to induce the transcription of a variety of T cell activation genes including the lymphokines we have studied (*9, 18, 19*). It also appears that the anti-CD28 stimulus does not exert its effect on lymphokine gene expression by altering the transcription of these genes.

The possibility that stimulation with anti-CD28 might regulate lymphokine gene expression by a posttranscriptional mechanism was investigated. Cells were treated with anti-CD3 or anti-CD3 together with anti-CD28 for 5 hours. We studied the stability of each of the specific mRNAs for

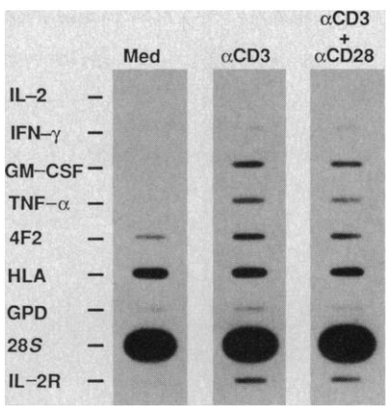


**Fig. 3.** Lymphokine gene transcription in peripheral blood T cells cultured in the presence of medium alone (Med), anti-CD3 immobilized to plastic, or anti-CD3 immobilized to plastic together with soluble anti-CD28. Cells were harvested 3 hours after stimulation. Nuclei were isolated, and run-on transcription assays were performed (*24*). The run-on transcription products were hybridized to filters containing 5 μg of plasmids with inserts specific for IL-2, IFN-γ, GM-CSF, TNF-α, 4F2HC (4F2), HLA, glyceraldehyde-3-phosphate dehydrogenase (GPD), 28*S* ribosomal RNA (28*S*), and IL-2 receptor (IL-2R) (*23*). The data are representative of two independent experiments, each performed in duplicate. Similar results were also obtained in cells treated for 5 hours.

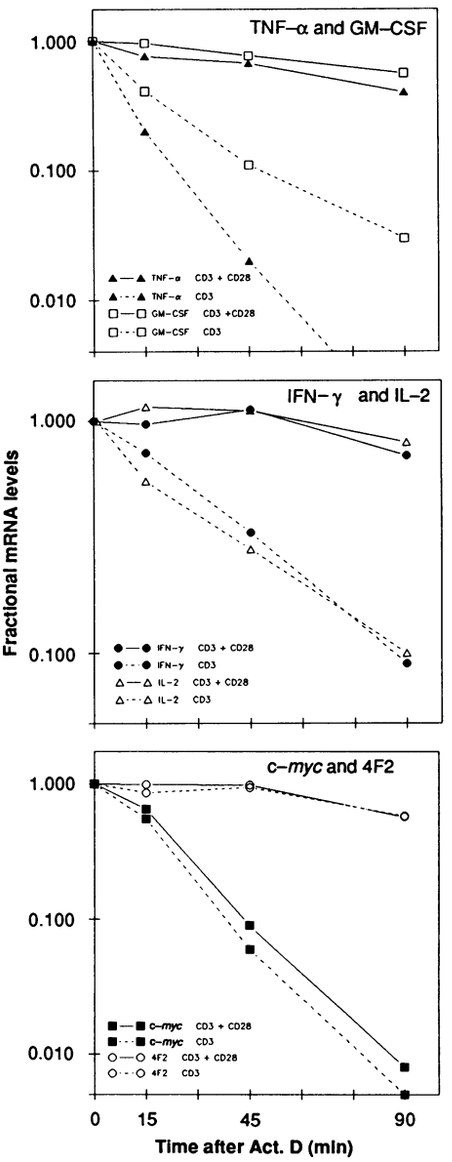


**Fig. 4.** The rate of mRNA degradation in T cells that had been stimulated for 5 hours with anti-CD3 in the presence or absence of anti-CD28. The rate of mRNA degradation of TNF-α, GM-CSF, IFN-γ, IL-2, c-*myc*, and 4F2HC (4F2) was determined by measuring the hybridization intensities of gene-specific probes (*6, 23*) to RNA blots containing mRNAs isolated from cells at the indicated times after the addition of actinomycin D (Act. D) (10 μg/ml). The amount of mRNA at the various times is expressed as a fraction of the mRNA level at time zero. The amount of mRNA was determined by scanning densitometry (*6*). The data presented are representative of three independent experiments.

TNF-α, GM-CSF, IFN-γ, IL-2, c-*myc*, and 4F2HC by measuring the levels of mRNA in cells harvested after the addition of 10 μg of actinomycin D per milliliter (Fig. 4). Costimulation of CD28 led to an increase in the stability of each of the four lymphokine mRNAs. In contrast, c-*myc* mRNA was unstable under both stimulation conditions and 4F2HC mRNA was a relatively stable transcript under both conditions. Thus, stimulation with anti-CD28 specifically stabilized the mRNAs of the four lymphokine genes studied.

As mentioned above, expression of the TNF-α gene after stimulation with anti-CD3 does not require new protein synthesis. Furthermore, cycloheximide can stabilize TNF-α mRNA and thus enhance its expression (*20*). To compare the effects of stimulation with anti-CD28 on mRNA stability to those of cycloheximide in T cells activated with anti-CD3, T cells were stimulated with anti-CD3, anti-CD3 together with anti-CD28, or anti-CD3 with cycloheximide (Fig. 5). With anti-CD3 alone, both TNF-α and c-*myc* mRNAs were rapidly lost from the cells after actinomycin D–induced transcriptional blockade. Costimulation of cells with anti-CD3 and anti-CD28 induced stabilization of the TNF-α mRNA but did not affect the stability of the c-*myc* mRNA. In contrast, when cells stimulated with anti-CD3 were cultured in the presence of cycloheximide, the mRNA stability of both TNF-α and c-*myc* was significantly enhanced. Thus, the alteration in mRNA stability induced by CD28 stimulation is much more specific than the stabilization of mRNA degradation that occurs in the presence of cycloheximide. The degradation of the 4F2HC and HLA mRNAs was not significantly affected by any of the culture conditions.

Our data show that the CD28 pathway augments T cell expression of several lymphokine genes by specifically stabilizing their mRNAs. The data also suggest that the ability of CD28 stimulation to stabilize mRNA results from a property shared by the four lymphokine mRNAs. One structural characteristic that is shared by all four lymphokine mRNAs is a conserved and homologous AU-rich sequence in their 3′ untranslated regions (*7, 21*). One common feature of these AU-rich sequences is that all have at least three reiterated copies of the sequence AUUUA. It has been shown that a sequence derived from the 3′ untranslated region of the GM-CSF gene, when genetically engineered into the 3′ end of a stable mRNA transcript such as β-globin, confers instability to the mature mRNA (*7*). A minimum of three reiterated AUUUA sequences (AUUUAUUUAUUUA) appears to be sufficient to produce instability in synthetic constructs (*22*). Whereas the presence of the conserved AU-rich sequences in the 3′ untranslated region of the above lymphokine mRNAs suggests that these sequences may play a role in regulating stability, such AU-rich sequences are not necessarily the CD28-responsive element. Both c-*myc* and c-*fos* mRNAs have AU-rich 3′ untranslated regions with at least three dispersed AUUUA sequences, and these mRNAs are not stabilized by the CD28 activation pathway. These findings suggest that either this sequence element is not sufficient to confer mRNA stability in the presence of CD28 stimulation or that the structural relationship of the reiterated sequence elements is important in conferring stability.

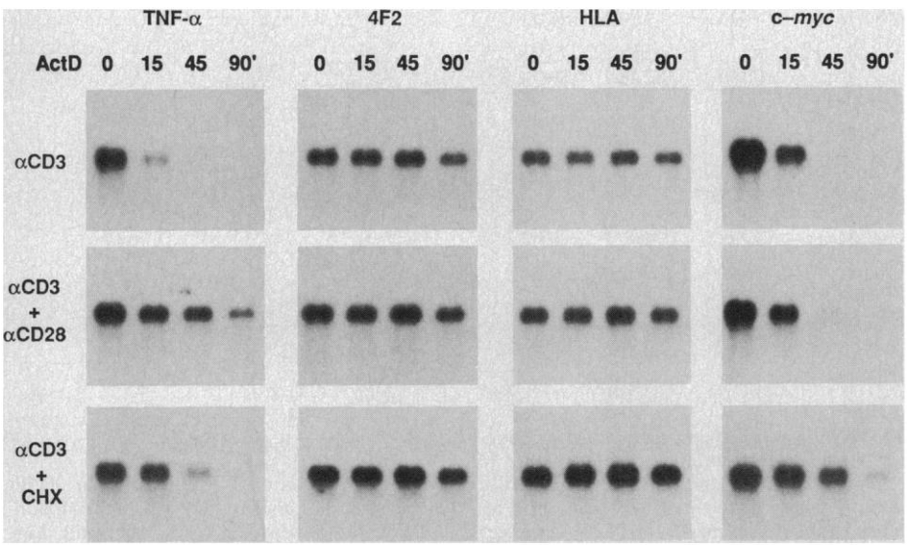


**Fig. 5**. The stability of TNF-α, 4F2HC (4F2), HLA, and c-*myc* mRNAs after treatment of peripheral blood T cells with anti-CD3, anti-CD3 together with anti-CD28, or anti-CD3 plus cycloheximide (CHX). $CD28^+$ T cells were cultured for 3 hours in the presence of anti-CD3 or anti-CD3 together with anti-CD28 as described in the legend to Fig. 3. To one set of cells treated with anti-CD3, cycloheximide was added at 10 μg/ml for the last 2 hours of stimulation. Actinomycin D (ActD) (10 μg/ml) was then added to the cultures, and cells were harvested after the times shown (minutes). Total cellular RNA was isolated and RNA blots were prepared from equalized samples of RNA. The blots were hybridized sequentially with TNF-α, 4F2HC (4F2), HLA, and c-*myc* gene-specific probes (*23*). For each condition, equivalent intensities of hybridization at the zero time point were used to allow direct comparison of the results. The data presented are representative of three independent experiments.

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23. The gene-specific probes used to probe RNA blots were isolated from low melting point agarose gels after digestion of the plasmids in which they were propagated with appropriate restriction endonucleases. Run-on transcription assays were performed with plasmids containing gene-specific inserts denatured by alkali treatment and transferred to nitrocellulose filters in a slot blot apparatus. The IL-2 probe was a 1.0-kb Pst I cDNA cloned into pBR322 (provided by S. Arya). The IFN-γ probe was a 1.0-kb Pst I cDNA fragment cloned into pBR322 (obtained from H. Young). The TNF-α probe was a 0.5-kb Eco RI–Bam HI cDNA fragment (provided by S. Kunkel). The GM-CSF probe was a 0.7-kb Eco RI–Hind III fragment (provided by S. Emerson). The 4F2HC probe was a 1.9-kb Eco RI cDNA fragment in pUC18 (provided by J. Leiden). The HLA probe was a 1.4-kb Pst I fragment from the HLA-B7 gene in pGEM-4. The c-*fos* probe was a 0.9-kb Sca I–Nco I fragment of pc-*fos*-1 available from the American Type Culture Collection. The c-*myc* probe was a 1.0-kb Cla I–Eco RI fragment isolated from a human c-*myc* cDNA (provided by D. Bentley). The IL-2 receptor probe was a 1.9-kb Eco RI–Bam HI fragment specific for the 55-kD IL-2 receptor subunit derived from pIL-2R3 (obtained from W. Greene).
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# The Role of Cis-Acting Promoter Elements in Tissue-Specific Albumin Gene Expression

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**The mouse albumin gene promoter has six closely spaced binding sites for nuclear proteins that are located between the TATA motif and nucleotide position −170. In vitro transcription with liver or spleen nuclear extracts of templates containing either mutated or polymerized albumin promoter elements establishes a hierarchy of the different protein binding sites for tissue-specific albumin gene transcription. The HNF-1 and C/EBP binding sites strongly activate transcription in a tissue-specific manner. The NF-Y binding site has a lower activation potential and is less specific, being equally efficient in liver and spleen nuclear extracts. The remaining elements are relatively weak activator sites.**

THE ACTIVITY OF MOST TISSUE SPECIFICALLY expressed mRNA genes is controlled primarily at transcription initiation (*1*). Three types of cis-acting elements may regulate the efficiency of this process: chromatin openers (*2*), enhancers (*3*), and promoters (*4*). Chromatin openers may decondense chromatin domains from a repressed to a potentially active state, thus making the gene or genes accessible to the transcription machinery. Enhancers can increase transcription initiation frequency from a distance in a location- and orientation-independent manner. Promoters are bipartite structures consisting of a core promoter and an upstream regulatory region. The core promoter (generally a TATA box, or equivalent element, and cap site) guides RNA polymerase II to the correct start site. The upstream regulatory region comprises one to several elements that can augment or decrease initiation frequency. Structural and functional studies on many promoters and enhancers have indicated that both have a modular structure and that some modules can be shared between the two. Both may therefore be assembled into a preinitiation complex that can be recognized efficiently by the transcription machinery, and this assembly may be mediated by trans-acting transcription factors that recognize specific DNA sequence elements with one functional domain and general components of the transcription apparatus such as RNA polymerase II and factor TFIID with another domain (*5*).

The serum albumin gene may need tissue-specific chromatin opener, enhancer, and promoter elements for its developmental activation during liver differentiation (*6*). In transient transfection studies, however, a relatively short 5′ flanking segment of about 150 nucleotides was sufficient to direct hepatocyte-specific transcription (*7*). We have also found that this sequence is necessary and sufficient to confer tissue-specific in vitro transcription to a G-free cassette (synthetic DNA fragment that does not contain any guanosine residue in its noncoding strand) reporter gene (*8*). As our in vitro system consists of histone-free nuclear extracts (NEs) and plasmid DNA templates, this differential in vitro transcription does not seem to need complex chromatin structures; more likely, it is the direct result of trans-acting tissue-specific transcriptional activators. We therefore established an inventory of DNA binding proteins that interact with albumin gene promoter elements, both for liver, the expressing tissue, and spleen, a nonexpressing tissue (*9–11*). In agreement with the results of other groups (*12*), the identified DNA binding proteins include the liver-specific or liver-enriched factors HNF-1 (*13*), C/EBP (*14*) and DBP (*10*), several CTF/NF-1–related factors (*15*), and the ubiquitous CAAT-factor NF-Y (*16*) (Fig. 1).

Results are now presented of two experimental strategies that were chosen to evaluate the relative importance of the protein-binding promoter elements in conferring liver-specific transcription of the albumin gene. In the first approach each individual binding site [with the exception of site F, whose deletion had only a marginal impact on transcription (*8*)] was destroyed by a short substitution of 11 or 13 nucleotides with unrelated DNA (Fig. 2A). The resulting templates contained the mutated promoters PΔA to PΔE, each having lost the capacity to interact with the cognate factor for the mutated site (*17*). The mutagenized templates, together with templates containing the wild-type promoter ($P_{wt}$) and the core promoter (PΔ-35, which consists of the TATA motif and the cap site) were subjected to transcriptional analysis with liver and spleen NEs (Fig. 2B).

In each in vitro transcription reaction, AdML200, a template containing the adenovirus major late promoter, was used as an internal standard. This promoter is recognized with a similar efficiency in both liver and spleen NEs (*8*). The relative transcriptional potential of the albumin promoter in liver and spleen was evaluated by dividing the radioactivity associated with the albumin transcript by the one associated with the adenovirus major late transcript. This ratio suggests that the albumin promoter is approximately 50-fold as active in liver as in spleen.

In liver NE, substitution of site B had the greatest effect on transcription, reducing the transcription efficiency approximately tenfold. In contrast, this same mutation had no significant effect on transcription in spleen NE. This result is consistent with that of a binding assay (Fig. 3B), which demonstrates that the cognate factor for this site, HNF-1, is liver-specific. In addition to the HNF-1 complex, a number of less abundant, more rapidly migrating complexes were observed with both liver and spleen

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