Functional Disruption of the CD28 Gene Transcriptional Initiator in Senescent T Cells*

Abbe N. Vallejo, Cornelia M. Weyand, and Jörg J. Goronzy‡

From the Departments of Medicine and Immunology, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Received for publication, June 22, 2000, and in revised form, November 6, 2000
Published, JBC Papers in Press, November 7, 2000, DOI 10.1074/jbc.M005503200

We recently reported that aging is accompanied by the emergence of CD4+CD28null T cells, a functionally aberrant lymphocyte subset rarely seen in individuals younger than 40 years. Here, we directly examined whether the lack of CD28 expression is due to a defect at the level of transcriptional initiation. Molecular studies reveal that CD28 gene transcription is controlled by two sequence motifs, sites α and β. In vitro transcription assays using initiator-dependent DNA templates revealed that reversed polarity or the deletion of either motif inhibited transcription, indicating that α/β sequences constitute a composite initiator. Moreover, nuclear extracts from CD28null cells failed to activate transcription of αβ-initiator DNA templates. Transcription of such templates was, however, restored with the addition of extracts from CD28+ cells. Although previously described initiator elements have been defined by a consensus sequence, the αβ-initiator has no homology to such sequence. These studies demonstrate that initiators have functions other than positioning elements for the basal transcription complex. Rather, initiators can have a direct role in regulating the expression of specific genes. The gain or loss of initiator activity can be an important determinant of cell phenotypes.

The cellular and molecular processes underlying immune processes during normal aging are complex. The immune system undergoes a constant turnover of cells and is highly dependent on the replenishment of new precursor cells. However, de novo production of T cells rapidly declines with the progressive involution of the thymus with age (1, 2). Consequently, there is replicative stress resulting in the progressive shortening of telomeres of peripheral lymphocytes (3, 4). Replicative senescence is associated with altered patterns of gene expression (5). Among T cells, senescence is accompanied by a characteristic loss of CD28, predominantly among CD8+ T cells (6, 7) and to a lesser degree among CD4+ T cells (8). Interestingly, CD4+CD28null T cells have also been found in patients with chronic inflammatory syndromes, such as those seen in rheumatoid arthritis, Wegener’s granulomatosis, and coronary artery disease (9–11). A CD28null phenotype is stable. Neither the triggering of the T cell receptor (TCR) nor signals generated by pharmacologic agents such as phorbol ester and calcium ionophore that bypass the TCR can restore CD28 expression (8, 12, 13).

Because CD28 is the dominant costimulatory molecule required for T cell activation, proliferation, and effector function (14), elucidation of the molecular basis for CD28 deficiency is of paramount interest. Inasmuch as CD28null T cells uniformly lack specific mRNA of all known splice variants (8, 12, 15), we evaluated the hypothesis that the loss of CD28 is due to a transcriptional block. In previous work, we reported that CD28 expression is controlled by two sequences, sites α and β, that are surprisingly situated immediately downstream from the TATA box (8). Nuclear proteins that specifically bind to sites α and β are limited to lymphoid tissue, and their expression patterns are correlated with the presence or absence of CD28 on the surfaces of T and B cells (15). Moreover, random mutations in either site can sufficiently inactivate promoter activity in reporter gene bioassays (8). The functional relevance of these sequence motifs is further indicated by the modulation of αβ-nuclear protein binding profiles by TCR triggering and during replicative senescence, two conditions that induce down-regulation of CD28 on the T cell surface (15).

Our finding that sites α and β map downstream from the TATA box (8) suggests that the expression of CD28 might be controlled at the level of transcriptional initiation. Although the TATA box has been traditionally considered as the assembly site of the basal transcription complex, flanking initiator (INR) sequences have been found to be critical core promoter elements (16–19). In TATA-containing promoters, such INRs are thought to be positioning elements that tether TATA-binding protein, ensuring the fidelity of transcription from the TATA box (20). INR-binding proteins may also interact with TATA-binding protein-associated factors, resulting in improved efficiency of transcription, as has been demonstrated for immunoglobulin promoters (21, 22). Although the existence of distinct INR-binding proteins is not clear, several proteins have been implicated in the activity of INRs. These include general transcription factors such as transcription factor II-I (23, 24) and the components of transcription factor IID (25–27), or regulatory proteins such as YY1 (28) and USF (29, 30). In the present work, the role of sites α and β as INRs was evaluated. Although these sequences have no homology with the consensus INR (18), sites α and β coincide with the putative transcription start site (31).

EXPERIMENTAL PROCEDURES

Cell Culture—T cell lines and clones were established from peripheral blood as described previously (8, 15, 32). Briefly, CD4+CD28null and CD4+CD28+ T cells were isolated from blood mononuclear cells by standard fluorescence-activated cell sorting procedures. Cells were

PCR, polymerase chain reaction; TdT, terminal deoxynucleotidyl transferase.
stimulated with anti-CD3 (OKT3, ATCC, Manassas, VA) and γ-irradiated autologous monocytes for 24 h. Subsequently, cells were subjected to limited dilution cloning in 96-well plates with feeder cells consisting of γ-irradiated, neuraminidase-treated EBV-transformed B lymphoblastoid cells without additional stimulation. Clones were isolated, and phenotypes were ascertained by immunofluorescence staining and flow cytometry (see below).

Primary CD4+ T cell lines were derived from unfraccionated blood mononuclear cells that were similarly stimulated with anti-CD3. After 24 h, CD4+ T cells were isolated by the immunodepletion of CD8+ cells using the VarioMacs system (Biotec Miltenyi, Auburn, CA). Purity of the isolated cells was assessed by flow cytometry. Cells were cultured on EBV-transformed B cell feeders and 20 units/ml recombinant human interleukin 2 (Proleukin, Chiron, Emeryville, CA). Sublines of CD4+CD28+ and CD4+CD28- T cells were subsequently established by fluorescence-activated cell sorting. All lines and clones were maintained by weekly stimulation with EBV-transformed B cell feeders and recombinant human interleukin 2 in a humidified 7.5% CO2 incubator as described previously (8, 15).

The T cell lymphoma lines Jurkat and HUT78 (ATCC) were maintained at densities of about 5 × 10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum. HUT78 was cultured in the presence of 20 units/ml recombinant human interleukin 2. Cells were maintained in a humidified 5% CO2 incubator.

In vitro Transcription—T cells were expanded for cell surface expression of CD3, CD4, and CD28 by three-color immunofluorescence staining and flow cytometry. The lack of CD28 expression in T cell clones or lines was also verified by reverse transcription-polymerase chain reaction (reverse transcription-PCR) procedures for the splice variants of CD28 (12, 15).

Clonality of the T cell clones was established by standard nested reverse transcription-PCR for the BV-BJ segments of the TCR. PCR products were cloned into the TA vector and recombinants were used to transform One-Shot™ Escherichia coli (Invitrogen, Carlsbad, CA). Sequencing of plasmids prepared from at least three randomly selected bacterial colonies authenticated the clones.

Nuclear Extracts—Nuclear extracts were prepared using a high salt extraction protocol described previously (9, 33). Extracts from the primary T cell lines and clones were prepared between 3 and 5 days after the last stimulation. Extracts from Jurkat and HUT78 cells were prepared during logarithmic growth. Protein concentrations of the extracts were determined by the Bradford method using a protein assay kit (Bio-Rad). Nuclear extracts were aliquoted, snap frozen in liquid nitrogen, and stored at −70 °C.

In Vitro Transcription Assay—Sequences of the human CD28 gene sites a and b, CTTGTATACCCTGGATAAGATCGCACTGAGGATCGCTTTGGTTTGGATGCGCTTTGAT (the underlined 5′ and 3′ sequences correspond to a and b, respectively) (8) were cloned into plasmid templates (provided by Dr. Jörg Kaufmann, Chiron Corp.) containing a 180-base pair G-less cassette downstream from a consensus TATA and the INR of terminal deoxynucleotidyl transferase (TdT) (34, 35). Sites a and b were introduced into these plasmids as separate elements. The contiguous unit replacing TdT-INR by the gene soeing technique (36).

The INR sequences (reverse transcription-PCR) procedures for the splice variants of the CD28 gene sites a and b, and the G-less cassette. Two clones of each construct were sequenced of plasmids prepared from at least three randomly selected bacterial colonies authenticated the clones.

Nuclear extracts were prepared using a high salt extraction protocol described previously (9, 33). Extracts from the primary T cell lines and clones were prepared between 3 and 5 days after the last stimulation. Extracts from Jurkat and HUT78 cells were prepared during logarithmic growth. Protein concentrations of the extracts were determined by the Bradford method using a protein assay kit (Bio-Rad). Nuclear extracts were aliquoted, snap frozen in liquid nitrogen, and stored at −70 °C.

In Vitro Transcription Assay—Sequences of the human CD28 gene sites a and b, CTTGTATACCCTGGATAAGATCGCACTGAGGATCGCTTTGGTTTGGATGCGCTTTGAT (the underlined 5′ and 3′ sequences correspond to a and b, respectively) (8) were cloned into plasmid templates (provided by Dr. Jörg Kaufmann, Chiron Corp.) containing a 180-base pair G-less cassette downstream from a consensus TATA and the INR of terminal deoxynucleotidyl transferase (TdT) (34, 35). Sites a and b were introduced into these plasmids as separate elements. The contiguous unit replacing TdT-INR by the gene soeing technique (36).

Templates containing a reversed orientation of aβ were also made. Constructs were amplified in E. coli DH5α (Life Technologies, Inc.) by standard transformation procedures and randomly selected bacterial colonies were screened for recombinant plasmids by PCR using primers specifically designed to detect the inserted a and/or b β sequence. Where PCR amplification of aβ was indicated, plasmids were prepared by a commercially available kit (EndoFree plasmid kit, Qiagen, Valencia, CA) and subjected to DNA sequencing of the entire the region spanning the INR of terminal deoxynucleotidyl transferase (TdT) (34, 35). Sites a and b were introduced into these plasmids as separate elements.

RESULTS

Sites a and β Constitute a Bona Fide INR Element—We have shown previously that two sequence motifs, sites a and β, regulate the constitutive expression of CD28 (8). The peculiar topographic location of these sequences immediately flanking the TATA box suggested that they might directly interact with the basal transcription complex. To evaluate this hypothesis, we adapted an in vitro transcription system (35) that measures transcription of a DNA cassette controlled by a consensus TATA box and TdT-INR. As shown in Fig. 1, replacement of the TdT-INR element of the DNA template by the CD28 gene aβ sequence resulted in the production of cassette transcripts in the presence of nuclear extracts from Jurkat, a T cell lymphoma that expressed high levels of CD28 (15). The levels of aβ-dependent transcription increased with the amounts of nuclear extract added in a manner similar to those seen with templates containing the TdT-INR. Consistent with previous studies (34, 35), the mutated variant of the TdT-INR yielded levels of cassette transcripts that were consistently and significantly lower than that seen with wild-type TdT-INR. Presumably, the low amounts of transcripts seen with the mutant TdT-INR represented the basal level of transcription from the upstream canonical TATA box.

Verification of aβ-Specific and TdT-INR Activating Proteins—Nuclear extracts used in the in vitro transcription assays were tested for
amounts of αβ-driven transcripts were equivalent to those seen with the wild-type TdT-INR.

In TATA-containing promoters, INRs are known to synergize with the TATA box, resulting in levels of transcription that are significantly higher than those seen with INR or TATA alone (38). This synergy is distinguished from a classical enhancer in that the latter induces transcription regardless of its orientation or distance from the basal complex assembled on the TATA box. Thus, we examined whether a reversed polarity, from α → β to β → α, affects INR activity. As shown in Fig. 3, two independent clones of DNA templates containing the reversed β → α sequence indeed yielded only low amounts of cassette transcripts. In these template constructs, the levels of transcription were equivalent to those produced by constructs containing the mutant TdT-INR.

αβ-INR in CD28null T Cells Is Nonfunctional—In previous studies, α/β-specific complexes were found to be uniformly lacking in CD4+ CD28null T cells. Gel shift assays revealed that neither contiguous αβ (8) nor separate α and β (15) probes showed protein binding activities with nuclear extracts from CD28null cells. Therefore, we examined whether this lack of DNA-protein complexes correlates with the absence of transcriptional activity. As shown in Fig. 4, in vitro transcription assays using nuclear extracts from various activated CD28+ T cells yielded cassette transcripts from DNA templates containing αβ as the INR element. The amounts of transcripts produced were equivalent to those seen with Jurkat nuclear extracts. In contrast, none of the nuclear extracts from CD28null T cells elicited transcription above basal levels. Extracts from CD28+ and CD28null T cells were, however, indistinguishable in their ability to promote transcription of templates containing the TdT-INR. As expected, templates with the mutated TdT-INR produced equivalent low/basal amounts of transcripts regardless of the CD28 phenotype of the extracts used.

The lack of αβ-INR activity in CD28null T cells could be due to the absence of αβ-specific transcription factors or to the presence of an inhibitor of αβ-proteins. To address this issue, reconstitution experiments were conducted using nuclear extracts from Jurkat and HUT78 cells as prototypes of CD28+ and CD28null T cells, respectively. As shown in Fig. 5, transcription of two DNA templates containing the αβ-INR was at low/basal levels with HUT78 extracts. However, the addition of increasing amounts of Jurkat extracts effectively restored αβ-mediated transcription. The reconstitution of transcriptional activity was proportional to the amounts of Jurkat extracts added to the reaction. Such mixtures of Jurkat and HUT78 extracts did not alter the levels of transcription of templates containing TdT-INR.

Reciprocal experiments were also conducted wherein HUT78 extracts were added in increasing amounts to a constant amount of Jurkat extracts. As shown in Fig. 6, the addition of HUT78 extracts did not affect αβ-driven transcription in Jurkat extracts. Transcription from TdT-INR templates were also unaffected by the titration of HUT78 extracts.

αβ-INR Activity Requires Coordinate Expression of Motif-
specific Transcription Factors—The observation that αβ-INR activity required α and β sequences in tandem (Fig. 2) suggested that nuclear proteins binding to both motifs are essential to transcription. Although these αβ-binding proteins remain to be identified, previous studies indicate that α- and β-complexes are distinct from each other (8). Therefore, we examined the effect of depletion of either complex in the efficiency of transcription. As shown in Fig. 7, incubation of Jurkat nuclear extracts in oligonucleotides corresponding to either site α or β resulted in the significant reduction of transcription of DNA templates containing the composite αβ sequences as INR.

The levels of αβ-driven transcription were effectively reduced to basal levels at oligonucleotide concentrations of 300 fmol. Similarly, incubation of extracts in oligonucleotides containing both α and β motifs also abrogated αβ-mediated transcription. As expected, none of the αβ oligonucleotides affected transcription of templates containing the TdT-INR.

**DISCUSSION**

The present work provides functional evidence for the direct role of sites α and β in CD28 gene transcription. Because the assay system adapted in this study stringently gauges the induction of INR-driven transcription over the basal activity of a canonical TATA box (34, 35), the ability of α and β to function as a core promoter element in a heterologous DNA template (Fig. 1) is impressive. Moreover, the lack of INR activity in similar templates with either sequence motif isolated from the TATA box (8) lends additional support to the significance of αβ motifs in CD28 gene expression. A classic example is the regulation of the somatomammotropin promoter (59), the interferon-responsive gene (57, 58) in which the INR can discriminate between two divergent sequences. Among the INRs served among eukaryotes (50, 51), there is an increasing body of evidence for INRs with divergent sequences. Among the evidence are the INRs of retinoic acid receptor β2 (37), mRNA cap-binding protein eIF4E (52), HIV-1 long terminal repeat (53), somatostatin receptor II (54), and vascular endothelial growth factor receptor (55). The second feature of αβ-INR is that sites α and β are nonoverlapping binding sites of discrete protein complexes (8, 15). Although the binding of motif-specific transcription factors occurs independently, the cooperative interaction of α- and β-bound proteins is required for transcriptional initiation. Indeed, the depletion of either α- or β-binding proteins from nuclear extracts by decoy motif-specific oligonucleotides abolishes αβ-INR activity (Fig. 7).

These peculiar properties of αβ-INR suggest that it may be a novel core promoter element. Interestingly, previous studies showed that α- and β-binding factors are found only in lymphoid tissue (15) supporting the notion that αβ-INR might account for the restricted expression of CD28 to T cells and some transformed B cells. Although the INRs are primarily involved in enhancing nucleation of the basal transcription complex on the TATA box (20, 56), there is also evidence for their accessory role in regulating expression of specific genes. A classic example is the regulation of the Drosophila alcohol dehydrogenase gene (57, 58) in which the INR can discriminate between two tandem promoters that are used differentially at various stages of development. Cell-specific gene expression is also increasingly indicated to be INR-dependent. The lymphocyte specificity of TdT is INR-dependent (46). The presence of cell type-specific INR-binding proteins, albeit unidentified, have been implicated in the maximal activation of the human chorionic somatomammotropin promoter (59), the interferon-responsive promoters such those of Fcε receptor 1b (60), guanylate-binding protein, and H-2Ld (61), and the cell-specific induction of the β2 isofrom of the retinoic acid receptor (37). Because of the divergent sequences of INRs of these latter genes and the αβ-INR from the consensus sequence, it is quite possible that cell type- or gene-specific INR-binding proteins profoundly influence the programs of gene expression. Thus, the identification of αβ-INR transcription factors is pivotal to understanding transcription assays in vitro do in fact reveal down-regulation or complete inhibition of transcription (49), providing physiological evidence for a direct link between INR function and cell/tissue phenotype.

**FIG. 7.** INR activity of αβ requires the coordinate binding of motif-specific proteins. About 5 μg of Jurkat nuclear extracts were incubated with varying concentrations (0, 40, 100, and 300 fmol) of double-stranded synthetic oligonucleotides (ds oligo) corresponding to site α (A), site β (B), or both, (AB), respectively, prior to the transcription assays. Assays with the wild-type (WT) or mutated form (MT) of the TdT-INR were conducted with control extracts (--) or those previously incubated with 300 fmol of A, B, or AB oligonucleotides.

![Fig. 6. Nuclear extracts from CD28<sup>−/−</sup> cells do not inhibit the αβ-INR promoting activity of extracts from CD28<sup>+</sup> cells.](http://www.jbc.org/) In experiments to those shown in Fig. 5, transcription assays with the TdT wild-type (WT) or mutant (MT) INR and αβ-INR constructs were conducted with 5 μg of nuclear extracts from Jurkat cells (CD28<sup>+</sup>) titrated with increasing amounts (5, 20, and 50 μg) of similar extracts from HUT78 cells (CD28<sup>−/−</sup>). In control reactions (--), 10 μg of nuclear extracts from either Jurkat or HUT78 cells were used.
the restricted expression of CD28.

The present data also unequivocally demonstrate that a CD4

The restricted expression of CD28.

The present data also unequivocally demonstrate that a CD4

The restricted expression of CD28.

The present data also unequivocally demonstrate that a CD4

The restricted expression of CD28.

The present data also unequivocally demonstrate that a CD4

The restricted expression of CD28.

The present data also unequivocally demonstrate that a CD4

The restricted expression of CD28.

The present data also unequivocally demonstrate that a CD4

The restricted expression of CD28.

The present data also unequivocally demonstrate that a CD4

The restricted expression of CD28.

The present data also unequivocally demonstrate that a CD4

The restricted expression of CD28.

The present data also unequivocally demonstrate that a CD4

The restricted expression of CD28.

The present data also unequivocally demonstrate that a CD4

The restricted expression of CD28.

The present data also unequivocally demonstrate that a CD4

The restricted expression of CD28.
58. Ren, B., and Maniatis, T. (1998) *EMBO J.* 17, 1076–1086
59. Jiang, S. W., Sheard, A. S., and Eberhardt, N. L. (1995) *J. Biol. Chem.* 270, 3683–3692
60. Eichbaum, Q. G., Iyer, R., Raveh, D. P., Mathieu, C., and Ezekowitz, R. A. (1994) *J. Exp. Med.* 179, 1985–1996
61. Wang, I. M., Blanco, J. C. G., Tsai, S. Y., Tsai, M. J., and Ozato, K. (1996) *Mol. Cell. Biol.* 16, 6313–6324
62. Park, W., Weyand, C. M., Schmidt, D., and Goronzy, J. J. (1997) *Eur. J. Immunol.* 27, 1082–1090
63. Vallejo, A. N., Mugge, L. O., Klimiuk, P. A., Weyand, C. M., and Goronzy, J. J. (2000) *J. Immunol.* 164, 2947–2954
64. Vallejo, A. N., Schirmer, M., Weyand, C. M., and Goronzy, J. J. (2000) *J. Immunol.*, 165, 6301–6307
65. Li, L. H., Nerlov, C., Prendergast, G., MacGregor, D., and Ziff, E. B. (1994) *EMBO J.* 13, 4070–4079
66. Philipp, A., Schneider, S., Vasrik, I., Finke, K., Xiong, Y., Beach, D., Altalal, K., and Eilers, M. (1994) *Mol. Cell. Biol.* 14, 4032–4043
67. Mai, S., and Martensson, L. L. (1995) *Nucleic Acids. Res.* 23, 1–9
