Differential Expression of Nur77 Family Members in Human T-Lymphotropic Virus Type 1-Infected Cells: Transactivation of the TR3/nur77 Gene by Tax Protein

XIAOLIN CHEN,1 VLADIMIR ZACHAR,1,2 CHAWNSHANG CHANG,3 PETER EBBESEN,1 AND XIANDONG LIU1*

Department of Virus and Cancer, Danish Cancer Society, DK-8000 Aarhus C, Denmark; Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic; and George H. Whipple Laboratory for Cancer Research, Departments of Pathology, Urology, and Biochemistry, University of Rochester, Rochester, New York 14642

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We analyzed the differential expression and regulation of three members of the Nur77 transcription factor family by the human T-lymphotropic virus type 1 (HTLV-1) Tax protein. We have demonstrated that in both HTLV-1-infected cells and Tax-expressing JPX-9 cells, TR3/nur77 is highly expressed, whereas neither NOR-1 nor NOT expression is detectable. Transient transfection analysis further confirmed the Tax transactivation of the TR3/nur77 promoter but not the NOR-1 promoter in different cell types. Furthermore, expression of a luciferase reporter gene driven by the NGFI-B (rat homolog of TR3/Nur77) response element (NBRE) provided evidence that Tax-mediated transactivation resulted in the induction of a functional protein. Cotransfection assays with the TR3/nur77 promoter sequence or the NBRE binding motif together with a series of Tax mutants have shown that Tax-induced TR3/nur77 expression is mediated by CREB/ATF-related transcription factors.

The human T-lymphotropic virus type 1 (HTLV-1) is an etiologic agent of adult T-cell leukemia/lymphoma (31) and tropical spastic paraparesis/HTLV-1-associated myelopathy (14, 30). Among all viral proteins, the most significant impact on the cellular biochemical machinery is exhibited by the 40-kDa regulatory phosphoprotein Tax (reviewed in references 11 and 43). Tax has been shown to be a powerful transactivator of viral long terminal repeats as well as diverse cellular genes. Tax exerts its effect by interacting with cellular transcription factors, which ultimately results in deregulation of a variety of gene promoters. Previous studies have in particular demonstrated that Tax can induce persistent expression of several immediate-early genes, such as c-fos, c-jun, egr-1, egr-2, and fra-1 (12, 13, 18, 37). These genes are involved in the control of normal cell growth and are modulated by a variety of growth-promoting agents including polypeptide growth factors, antigens, and diverse mitogens (19). In HTLV-1-infected cells, however, overexpression of immediate-early genes provides additional growth signals, which may thus lead to an aberrant cell proliferation and/or cell death.

The newly characterized immediate-early genes TR3 (human homolog of nur77, NGFI-B, N10, and TIS1; also termed NAK-1; in this report, designated as TR3/nur77), NOT (human homolog of Nur1 and RNR-1; also called TINUR), and NOR-1 (also called MINOR) encode three closely related transcription factors, which constitute a distinct Nur77 subfamily within the steroid/thyroid hormone receptor superfamily (4, 16, 20, 22, 25, 27–29, 32, 35). TR3/Nur77 has been shown to be induced in a variety of cells in response to signals for growth and differentiation. It is activated by mitogenic serum growth factors in fibroblasts (16, 32), nerve growth factor (NGF), membrane depolarization during neuronal differentiation of the pheochromocytoma cell line PC-12 (25, 42), and the T-cell receptor (TCR) signaling in immature thymocytes and T-cell hybridomas (21, 40). Two other members of the Nur77 family, NOR-1 and NOT, have extensive homology with TR3/Nur77, and they both can be induced in response to mitogenic stimulation in a variety of cell types (20, 22, 28). Despite their close relationship, the three members of the Nur77 family are differentially regulated, which indicates that they play distinct roles. In response to TCR stimulation, only TR3/Nur77 and NOR-1 appear to be rapidly induced to high levels in thymocytes and T-cell hybridomas, and moreover, constitutive expression of both TR3/Nur77 and NOR-1, but not NOT in T lymphocytes, leads to massive apoptosis and upregulation of CD25 (6).

The possibility that the transcription factors of the Nur77 family might be involved in the development of HTLV-1-related disease, we set out to investigate regulation of TR3/Nur77, NOR-1, and NOT expression by the Tax transactivator. Differential expression of Nur77 family members in HTLV-1-infected T cells. The Nur77 family members, TR3/Nur77, NOR-1, and NOT, have extensive homology in their DNA binding domains, zinc fingers, and A boxes. However, in the N-terminal transactivation domain, NOR-1 and NOT are only 21 and 27% homologous to the TR3/Nur77 protein, respectively. Similarly, NOR-1 and NOT display in their transactivation domains only 36% amino acid similarity (6). In order to circumvent the cross-hybridization in analyzing the expression of each of the Nur77 family members in HTLV-1-infected T cells, the TR3/nur77-, NOR-1-, and NOT-specific DNA probes were designed to correspond to different regions of the coding sequences.

To prepare the probes, TR3/nur77-, NOR-1-, and NOT-
specific sequences were amplified with a cDNA pool from human peripheral blood mononuclear cells stimulated by 50 ng of phorbol-12-myristate-13-acetate (TPA) per ml, 1 μg of calcium ionophore A23187 per ml, and 10 μg of cycloheximide per ml for 1 h. Primers specifically targeting each of the genes were used as follows: TR3/nur77 (5'-TCATGACCGCTACA CAG and 5'-GTAGGATGGAATAGCTC), NOR-1 (5'-CCT TGTCGAGCTTTAACAG and 5'-ACAGGCAGCTAAGG CTTGG), and NOT (5'-AACCTGACTATCAATGAGTG and 5'-CAATGAGGAAGGCGAAGAT). Amplified sequences (517 bp for TR3/nur77, 410 bp for NOR-1, and 352 bp for NOT) were cloned into the pCR2.1 vector (Invitrogen, NV Leek, The Netherlands) and verified by DNA sequencing. For Northern blot analysis, the total RNA was isolated by the acid phenol extraction method (9), and the samples amounting to 10 μg were fractionated in a formaldehyde agarose gel and transferred to a polyvinylidene difluoride N membrane (Millipore, Bedford, Mass.). In the control experiments, the random-primed hybridization probes specific for tax and GAPDH were prepared with the pSGTax (provided by M. Seiki, Kanazawa University, Kanazawa, Japan) and pHcGAP (provided by Feng Qiu, University of Heidelberg) plasmids, respectively. Hybridization was performed according to the standard protocol (34).

Under stringent hybridization conditions, we were able to detect high levels of TR3/nur77 transcripts in both of the HTLV-1-infected T-cell lines, C8166-45 and MT-2 (2, 15, 33); however, in Jurkat, Hut-78, and Molt-4, the T-cell lines negative for HTLV-1, expression of TR3/nur77 was undetectable (Fig. 1). Interestingly, in none of the tested cell lines did transcriptional expression of NOT and NOT take place, thus suggesting a differential regulation of the Nur77 family transcription factors in HTLV-1-infected cells.

**Induction of TR3/nur77 expression by Tax protein.** To further investigate whether the constitutive expression of TR3/nur77 is induced by Tax protein, we employed a Jurkat derivative cell line, JXP-9, in which the tax gene is under the control of an inducible metallothionein promoter (26). As shown in Fig. 1, induction of Tax by CdCl2 resulted in a rapid activation of TR3/nur77, with specific transcripts first observable at 4 h and increasing continuously afterwards. In contrast to TR3/nur77, NOT and NOT remained undetectable, similar to the situation in HTLV-1-transformed cell lines. Furthermore, mutated Tax induced by CdCl2 in the control cell line JXP/N failed to transcriptionally activate all three Nur77 family members (data not shown). These data thus provide evidence that Tax is responsible for the high level of TR3/nur77 expression in HTLV-1-infected cells.

**Tax-mediated transactivation of the TR3/nur77 promoter.** In order to analyze Tax transactivation, the TR3/nur77 promoter containing a 2,419-bp DNA sequence upstream of the transcription start site was cloned into the chloramphenicol acetyltransferase (CAT) reporter vector p-2149TR3CAT as described previously (38). The plasmid was cotransfected along with the Tax-expressing plasmid pCMV-Tax (36) or the control plasmid pCMV into the choriocarcinoma line JEG-3, the neuroblastoma line SK-N-SH, simian virus 40-transformed African green monkey cell line COS-7, and NIH/3T3 mouse cells (American Type Culture Collection, Manassas, Va.).

All transfection experiments were performed in six-well plates in triplicate with serum-free Optimum medium (Life Technologies, Paisley, Scotland). NIH/3T3 and COS-7 cells were transfected with Lipofectamine (Life Technologies) 24 h after the seeding of 3 × 10⁵ cells/well. For the transfection of JEG-3 and SK-N-SH, 4 × 10⁵ cells/well were grown for 24 h and transfected with Lipofectin (Life Technologies). The cultures were further maintained for 36 to 48 h, at which time the CAT quantitation was performed with the CAT enzyme-linked immunosorbent assay (ELISA) kit (Boehringer, Mannheim, Germany). The activities in individual samples were normalized on the basis of protein content, which was determined by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.).

Expression of Tax resulted in 5- to 195-fold induction of the CAT gene in different cell lines, suggesting that Tax can transactivate TR3/nur77 in a broad range of cell types (Fig. 2). In addition, we have also subcloned the NOT-1 promoter into the reporter vector and used it in parallel cotransfection experiments. In contrast to TR3/nur77, however, the NOT-1 promoter was only weakly stimulated by Tax, exhibiting less than 1/10 of the activity found with the TR3/nur77 promoter (data not shown). These data corroborate our previous analysis of transcriptional patterns as shown in Fig. 1.

**Activation of NGFI-B response element (NBRE) mediated by Tax protein.** Early work has shown that NGFI-B (rat homolog of TR3/Nur77) can regulate gene expression via specific interaction with NBRE, the AAAAGGTCA octameric non-
palindromic DNA element determined by genetic selection (39). Recently, Cheng et al. (6) have demonstrated that TR3/Nur77, NOR-1, and NOT can all bind to the NBRE motif and that the affinity of binding decreases from TR3/Nur77 to NOR-1. Because Tax can upregulate TR3/nur77 expression, the activation of the NBRE-mediated reporter gene is expected to take place as well.

To obtain experimental evidence, the firefly luciferase (Luc) reporter vectors containing one (pB1a-Luc) or eight [p(B1a)8-Luc] (41) NBRE consensus elements upstream of a minimal prolactin promoter of the pPro36-Luc vector (1) were used in cotransfection together with the Tax-expression plasmid (pCMV-Tax) in Jurkat cells. The method for transfection and detection of luciferase activity has been detailed previously (5). With the vector with a single NBRE, a more than 10-fold increase of luciferase gene expression was observed upon Tax transactivation, whereas in the case of the vector with eight NBREs the increase was more than 50-fold (Fig. 3). Similar activation of NBRE binding activity by Tax was also observed in several other cell types mentioned above (data not shown). These results thereby provide evidence that the functional TR3/Nur77 protein can be induced in HTLV-1-infected cells and that the Tax protein plays a role in modulation of the responses mediated by the TR3/Nur77 protein.

CREB/ATF-related transcription factors are possibly involved in Tax-induced TR3/Nur77 expression. Tax protein exhibits at least two discrete domains which are involved in separate transcriptional regulatory pathways. One of these pathways is mediated via the Rel-related family of kB enhancer-binding proteins, and the other is mediated via the CREB/ATF-related family of transcription factors (36). To explore which region of Tax protein is involved in the TR3/nur77 transactivation, we employed Tax mutants which were defective in the induction of the NF-kB pathway, M1 and M47 mutants are deficient in inducing the CREB/ATF pathway, and M7 is deficient in inducing both the NF-kB and CREB/ATF pathways. The transient expression of the CAT reporter gene was quantitated by the CAT ELISA after the samples were normalized for the protein content. Bars represent the means ± standard errors of the means of three replicates.}

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the regulation of Nur77 family members since the previous study of TCR engagement in the thymocytes and T-cell hybridomas suggested that TR3/Nur77 expression is tightly linked only with NOR-1 coexpression (6). Moreover, on the basis of experiments analyzing activation by Tax of the Nur77 family recognition motif NBRE, it appears that induction of TR3/ Nur77, or any other hitherto-unrecognized family member, occurs through the CREB/ATF-mediated pathway.

In recent years, ample evidence has been accumulated to suggest that the Tax transactivator is implicated in neoplastic cell transformation by inducing immediate-early genes. c-fos, fra-1, egr-1, and egr-2, all of which are under physiologic conditions transiently activated following antigenic or mitogenic stimulation of T cells, have previously been found to undergo sustained induction as a result of Tax transactivation (12, 13, 18, 37). In the present paper, we provide evidence that yet another immediate-early gene, TR3/nur77, is subject to regulation by Tax. However, it appears that the role of TR3/Nur77 in cell cycling may be complex. In contrast to c-Fos, Fra-1, Egr-1, and Egr-2, TR3/Nur77 has been found to play a role in the induction of T-cell apoptotic death (6). The TR3/Nur77 pathway thus could provide, in addition to those of tumor necrosis factor alpha (TNF-α) and Fas ligand, a more comprehensive explanation for apoptosis induced by Tax in T cells and murine fibroblasts (5, 7, 8, 10, 41).

TR3/Nur77 is not expressed in the prenatatal central nervous system (CNS) but is highly expressed in the adult rat brain in many areas including the olfactory bulb, cortex, basal ganglia, and hippocampus, suggesting its involvement in the development and maturation of specific sets of CNS neurons (44). Specific TR3/Nur77 expression can also be quickly induced by focal mechanical lesions of the brain, excitatory epileptogenic treatments, and in vitro depolarization of neural cells (17, 44). In particular, recent results have shown that the deregulation of TR3/Nur77 and its family members NOT and NOR-1 could possibly be involved in neuron degeneration in Parkinson’s disease (44). With respect to HTLV-1-associated CNS disease, it has been suggested that a central role in the etiopathogenesis of tropical spastic paraparesis/HTLV-1-associated myelopathy is played by highly activated HTLV-1-infected T cells secreting Tax protein (10). Interestingly, extra cellular Tax is able to modulate gene expression in a way similar to that of cytokines (3, 23, 24), and it has been shown to induce neurons to produce TNF-α in vitro (10). In addition to the proposed role for TNF-α in the development of neurodegenerative disorders, it is plausible that uncontrolled expression of TR3/Nur77 is also of importance. Our own data, with neuroblastoma cell line SK-N-SH, provide initial experimental evidence for possible involvement of this transcription factor in HTLV-1-related disorders of the CNS.

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REFERENCES


44. Zetterstrom, R. H., R. Williams, T. Perlmann, and L. Olson. 1996. Cellular expression of the immediate early transcription factors Nur1 and NGFI-B suggests a gene regulatory role in several brain regions including the nigrostriatal dopamine system. Mol. Brain Res. 41:111–120.