

Functional redundancy of the Nur77 and Nor-1 orphan steroid receptors in T-cell apoptosis

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The transcription factor Nur77 (NGFI-B), a member of the steroid nuclear receptor superfamily, is induced to a high level during T-cell receptor (TCR)-mediated apoptosis. A transgenic dominant-negative Nur77 protein can inhibit the apoptotic process accompanying negative selection in thymocytes, while constitutive expression of Nur77 leads to massive cell death. Nur77-deficient mice, however, have no phenotype, suggesting the possible existence of a protein with redundant function to Nur77. To explore this possibility, we have characterized the role of two Nur77 family members, Nurr1 and Nor-1, in TCR-induced apoptosis. We found that Nor-1 and Nurr1 can transactivate through the same DNA element as Nur77, and that their transactivation activities can be blocked by a Nur77 dominant-negative protein. In thymocytes, Nor-1 protein is induced to a very high level upon TCR stimulation and has similar kinetics to Nur77. In contrast, Nurr1 is undetectable in stimulated thymocytes. Furthermore, constitutive expression of Nor-1 in thymocytes leads to massive apoptosis and up-regulation of CD25, suggesting a functional redundancy between Nur77 and Nor-1 gene products. As in the case of our Nur77-FL mice, FasL is not detectable in the thymocytes of Nor-1 transgenic mice. Constitutive expression of Nur77 in *gld/gld* mice rescues the lymphoproliferative phenotype of the FasL mutant mice. Thus, Nor-1 and Nur77 demonstrate functional redundancy in an apparently Fas-independent apoptosis.

Keywords: apoptosis/Fas/FasL/NGFI-B/orphan steroid receptor

Introduction

During development in the thymus, immature thymocytes which express self-reactive T-cell antigen receptors (TCRs) are eliminated from the developing T-cell repertoire (for review, see Nossal, 1994; Robey and Fowlkes, 1994; von Boehmer, 1994, and references therein). This process of clonal deletion (negative selection) is thought to be mediated by apoptotic signals delivered to thymocytes which express TCRs having a strong affinity for self-peptide/major histocompatibility complexes (for review, see Allen, 1994). This mechanism is crucial for generating a peri-

pheral T-lymphocyte population with low potential auto-reactivity. Further safeguards also operate in the peripheral immune organs, where mature T cells with TCRs recognizing non-thymic self-antigen undergo apoptosis. Apoptosis in the peripheral organs of the immune system involves the Fas–FasL and tumor necrosis factor pathways (Krammer *et al.*, 1994; Lenardo *et al.*, 1995; Nagata and Golstein, 1995; Zheng *et al.*, 1995; Abbas, 1996).

Using a subtractive hybridization technique, we and others have identified Nur77 (NGFI-B, N10, Nak1; Hazel *et al.*, 1988; Milbrandt, 1988; Ryseck *et al.*, 1989; Nakai *et al.*, 1990) as a gene whose expression is induced rapidly by TCR signaling in immature thymocytes and T-cell hybridomas (Liu *et al.*, 1994; Woronicz *et al.*, 1994). Nur77 is classified as a member of the superfamily of nuclear steroid receptors due to sequence homology in the DNA-binding domain, and is an 'orphan' member because there is no known ligand for Nur77. It is possible that no ligand exists because Nur77 is transcriptionally active in many cell types (Paulsen *et al.*, 1992; Davis *et al.*, 1993). The Nur77 protein has the typical steroid receptor organization composed of an N-terminal transactivation domain, a central DNA-binding domain containing two zinc fingers, and a C-terminus with homology to hormone-binding domains (Carson *et al.*, 1990). Unlike most steroid receptors which bind DNA as dimers, Nur77 can bind as a monomer to an estrogen receptor half-site element containing two additional adenine nucleotides at the 5' end (NBRE 5'-AAAGGTCA-3') (Wilson *et al.*, 1991, 1993). Protein binding to the NBRE correlates well with the onset of apoptosis in T-cell hybridomas (Woronicz *et al.*, 1995). In the presence of retinoic acid, Nur77 can heterodimerize with the retinoic X receptor and bind to a direct repeat element of estrogen receptor half-sites separated by five nucleotides (Forman *et al.*, 1995; Pagano *et al.*, 1995; Perlmann and Jansson, 1995). Nur77 transcription is also induced in a variety of cells in response to signals for growth and differentiation. It is activated by mitogenic serum growth factors in fibroblasts (Hazel *et al.*, 1988; Ryseck *et al.*, 1989), or by nerve growth factor (NGF) during neuronal differentiation of the pheochromocytoma cell line PC12 (Milbrandt, 1988). In contrast to activation through the TCR signals, however, serum and NGF stimulation only cause transient and low level expression of Nur77.

In addition to its pattern of expression, several other lines of evidence suggest that Nur77 is involved in TCR-mediated apoptosis. Overexpression of a dominant-negative Nur77 protein can inhibit TCR-mediated apoptosis in T cell hybridomas (Woronicz *et al.*, 1994). The thymocytes of transgenic mice overexpressing a dominant-negative mutant Nur77 are protected from antigen-induced apoptosis (Calnan *et al.*, 1995; Zhou *et al.*, 1996). While expression of the dominant-negative

Nur77 does not result in any gross changes in T-cell development, constitutive expression of the full-length Nur77 gene product results in massive apoptosis. A substantial decrease in the number of thymocytes and peripheral T cells was observed in the full-length Nur77 transgenic mice (Calnan *et al.*, 1995; Weih *et al.*, 1996). Thus, experiments in both the hybridoma and mouse implicate Nur77 as playing an important role in TCR-mediated apoptosis.

Nur77-deficient mice have been generated (Lee *et al.*, 1995). In these mice, the process of negative selection and peripheral TCR-mediated apoptosis is normal (Lee *et al.*, 1995). Indeed, the Nur77^{-/-} mice exhibit no phenotype at all (Crawford *et al.*, 1995), suggesting that a protein with functional redundancy to Nur77 may exist. Two other Nur77 family members have been identified: Nurr1 (or Not1; Law *et al.*, 1992; Mages *et al.*, 1994) and Nor-1 (or MINOR; Ohkura *et al.*, 1994; Hedvat and Irving, 1995). Both Nurr1 and Nor-1 share extensive homology with Nur77 in their DNA-binding domains, zinc fingers and A box (92 and 91% respectively). The sequences of the three Nur77 family members diverge outside the DNA-binding domain. Nurr1 is 27 and 67% homologous to the Nur77 protein in its respective N- and C-terminal domains, while Nor-1 is 21 and 54% similar to Nur77 in the corresponding N- and C-terminal regions.

To explore the possibility that Nurr1 or Nor-1 may perform a similar function to Nur77 during TCR-mediated apoptosis, we have characterized Nurr1 and Nor-1 in T cells. We found that the transactivation activity by either Nor-1 or Nurr1 on an NBRE-based reporter construct can be observed in T cells and can be blocked by the presence of a Nur77 dominant-negative protein. In thymocytes and T-cell hybridomas, Nor-1 is induced rapidly in response to TCR signals with kinetics similar to that of Nur77. In contrast, Nurr1 is undetectable in stimulated thymocytes. Constitutive expression of Nor-1 leads to massive apoptosis in the thymocytes of transgenic mice without elevated FasL expression, a phenotype similar to our Nur77 transgenic mice. The Fas-independent pathway is confirmed further by the absence of lymphoproliferative disease in Nur77 *gld/gld* mutant mice. We conclude that Nor-1 and Nur77 share many characteristics in TCR-mediated apoptosis.

Results

Dominant-negative Nur77 inhibits the transactivation activity of all the Nur77 family members

In order to establish the potential role of the Nur77 family in TCR-mediated apoptosis, we sought to characterize the functional similarities or differences between the family members both *in vitro* and *in vivo*. The following properties were examined: transactivation and DNA-binding activity, the kinetics of induction during TCR-mediated apoptosis and the ability to initiate apoptosis in thymocytes.

To examine the transactivation activity of the Nur77 family members, we used a luciferase reporter construct under the control of the Nur77 DNA-binding element (NBRE; Woronicz *et al.*, 1995). All the Nur77 family members are expressed using the cytomegaloviral promoter expression plasmid pCI. Co-transfection of the

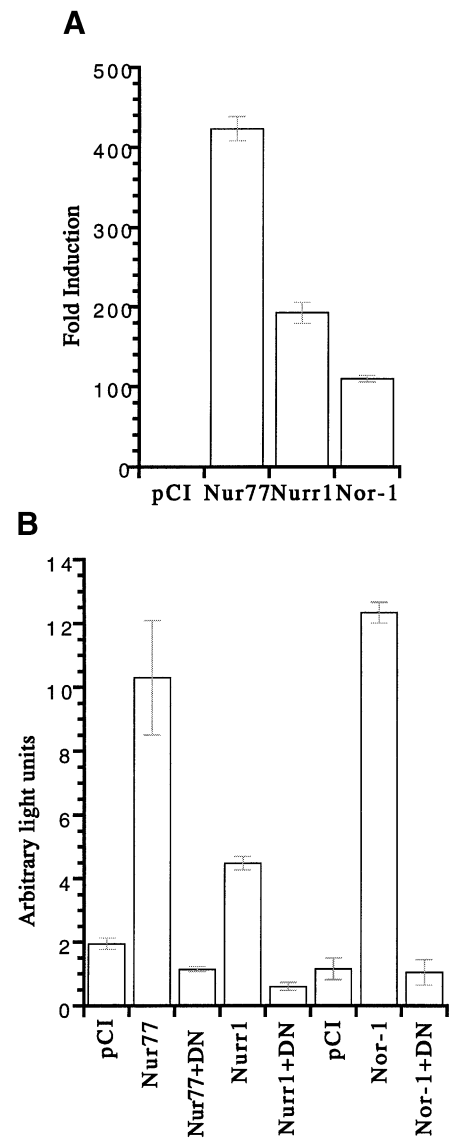


Fig. 1. Expression of dominant-negative Nur77 leads to inhibition of the transactivation activity of all the Nur77 family members.

(A) Jurkat T cells were transfected with luciferase reporter construct containing three copies of the NBRE along with one of the following plasmids: the eukaryotic expression plasmid pCI, pCI-Nur77 (Nur77), pCI-Nurr1 (Nurr1) or pCI-Nor-1 (Nor-1). Equimolar concentrations of each plasmid were used. Calf thymus DNA was added to adjust for the total amount of DNA between the samples. Cells were harvested 36–48 h after transfection and the luciferase activity was assayed as per the manufacturer's instructions. Fold induction is calculated relative to the background light units in the vector control sample (pCI). The values shown are the averages of three independent experiments plus the standard deviation. (B) Experiments similar to those described in (A) were performed, except that that a construct with two copies of NBRE was used for the Nur77 and Nurr1 transactivation assay. In some samples, a plasmid expressing the dominant-negative Nur77 was also added (+DN).

luciferase reporter construct with three NBRE sites along with the Nur77 expression plasmid leads to a >400-fold increase in the luciferase activity (Figure 1A). Similarly, in these experiments, both Nor-1 and Nurr1 can also transactivate through the NBRE with at least 100-fold activation over the background (Figure 1A). Although the degree of activation varies between experiments, Nur77 consistently has the highest transactivation activity, followed by Nurr1 and Nor-1.

One possible explanation for the different phenotypes observed between Nur77-deficient mice and Nur77 dominant-negative transgenic mice is that the dominant-negative protein is able to inhibit all the Nur77 family members. Indeed, co-expression of the Nur77 dominant-negative protein completely inhibits the transactivation activity of not only Nur77 but also that of Nurr1 and Nor-1 (Figure 1B). Thus, each of the Nur77 family members possesses very similar mechanisms of transactivation. Although there are differences in their efficiency of transactivation, all three proteins can be inhibited by the dominant-negative Nur77 protein.

Nor-1 displays a similar pattern of expression to Nur77 upon TCR stimulation, while Nurr1 is undetectable in stimulated thymocytes

In order to examine expression patterns of both Nurr1 and Nor1 during antigen-induced stimulation, antibodies were raised against Nurr1 and Nor-1. One problem that exists with a multigene family, however, is the potential for antibody cross-reactivity between the various family members. To circumvent this problem, antisera were raised against only the N-terminal transactivation domains of both Nurr1 and Nor-1. The transactivation domain of Nur77 displays only 21 and 27% homology to the corresponding regions of Nor-1 and Nurr1, respectively. Nurr1 and Nor-1 also display a lesser degree of homology to each other in their transactivation domains, with 36% amino acid similarity. The specificity of the Nor-1 and Nurr1 antisera was assessed via gel-shift assay with labeled NBRE DNA as a probe. Antisera from three different rabbits were tested with Nurr1 and Nur77 *in vitro* translated proteins (IVT Nurr1 and IVT Nur77). The antisera from rabbit 12-51 can supershift the Nurr1–NBRE complex but not the Nur77–NBRE complex (Figure 2A). Similarly, the anti-Nor-1 antisera can block Nor-1 binding to NBRE but not Nur77 binding activity (Figure 2B). Hence, the Nurr1 and Nor-1 antisera are each specific for themselves. An anti-Nur77 antiserum raised previously against the full-length Nur77 protein had a small cross-reactivity with Nor-1. Results with Nur77 have been confirmed previously using a monoclonal antibody specific for Nur77 (Woronicz *et al.*, 1995).

Using these antisera, we examined the expression pattern of the individual Nur77 family members in thymocytes and T-cell hybridomas stimulated with either anti-CD3 or a combination of phorbol ester (PMA) and calcium ionophore (ionomycin), which mimics TCR-mediated signaling. We have shown previously that the NBRE-binding complex is induced to a high level in response to either anti-CD3 or PMA/ionomycin signals in T-cell hybridomas and thymocytes (Woronicz *et al.*, 1994, 1995). This complex contains Nur77, as its binding activity was reduced significantly when anti-Nur77 antibodies were added to the reaction (Woronicz *et al.*, 1995). To see if Nor-1 and Nurr1 proteins were also part of the NBRE complex, the same experiment was repeated in the presence of the newly generated antibodies. Consistent with previous observations, NBRE-binding activity can be seen starting at 1 h post-stimulation in thymocyte extracts. Addition of either Nor-1 or Nur77 antisera leads to a dramatic decrease in the binding complex (Figure 3A). When added together, anti-Nur77 and anti-Nor-1 abolished

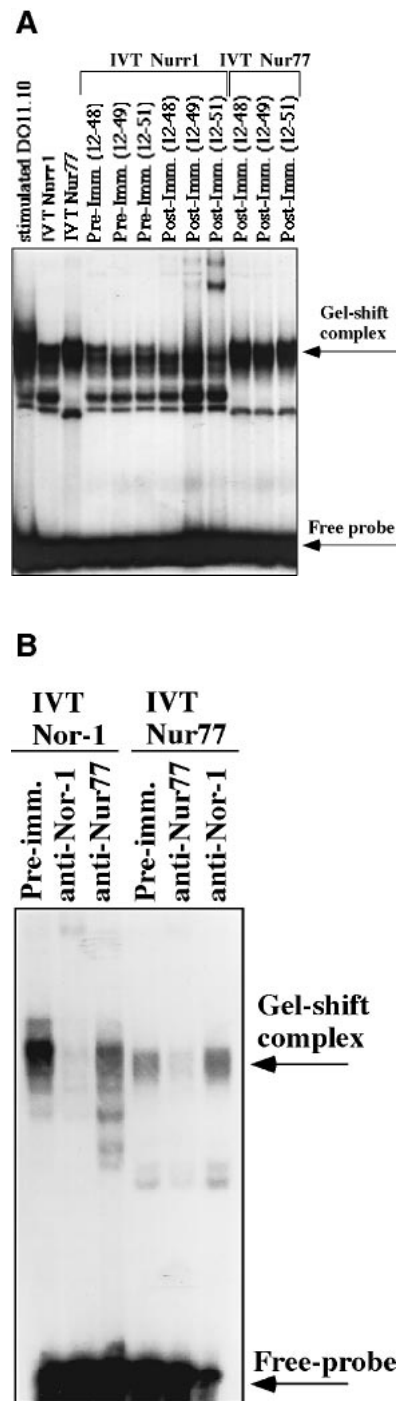


Fig. 2. Gel-shift analysis with NBRE as a probe to examine the cross-reactivity of the Nurr1 and Nor-1 antiserum with Nur77. (A) The Nurr1 antiserum does not cross-react with Nur77. Gel-shift analysis was done using the NBRE as a probe and Nurr1 *in vitro* translated protein (IVT Nurr1) or Nur77 *in vitro* translated protein (IVT Nur77) in the presence of pre-immune serum (Pre-Imm.) or Nurr1 antiserum (Post-Imm.). The numbers 12-48, 12-49 and 12-51 indicate antisera from different rabbits. Nuclear extract from DO11.10 hybridoma stimulated with PMA/ionomycin was used as a positive control. (B) Similar experiments to those described in (A) were performed, except that Nor-1 antisera (α -Nor-1) or Nur77 antisera (α -Nur77) and *in vitro* translated Nor-1 (IVT Nor-1) or Nur77 (IVT Nur77) were used.

all of the NBRE DNA–protein binding activity (Figure 3A). Anti-Nurr1 antisera, however, did not supershift the NBRE-binding complex from the thymocyte extracts

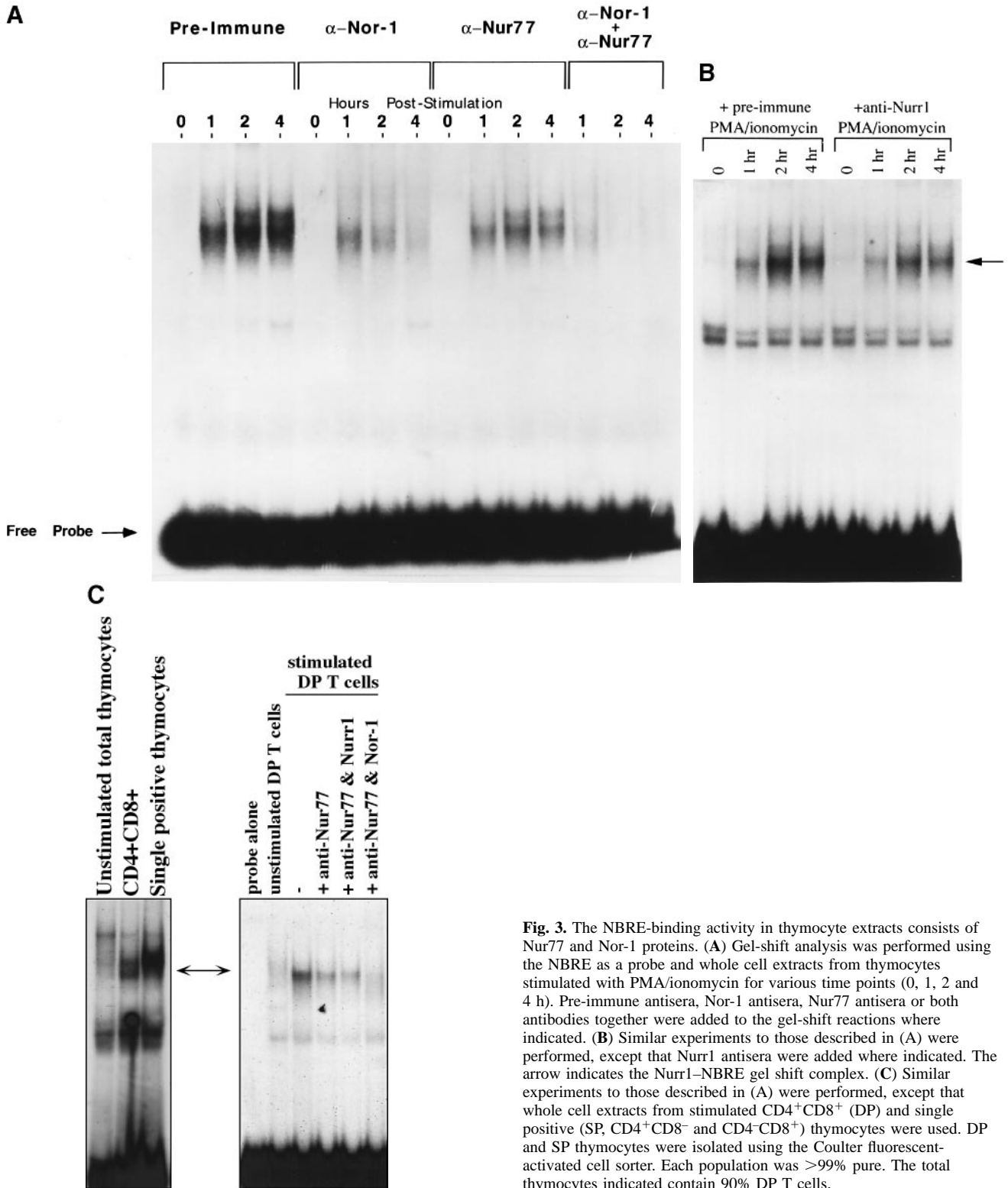


Fig. 3. The NBRE-binding activity in thymocyte extracts consists of Nur77 and Nor-1 proteins. (A) Gel-shift analysis was performed using the NBRE as a probe and whole cell extracts from thymocytes stimulated with PMA/ionomycin for various time points (0, 1, 2 and 4 h). Pre-immune antisera, Nor-1 antisera, Nur77 antisera or both antibodies together were added to the gel-shift reactions where indicated. (B) Similar experiments to those described in (A) were performed, except that Nurr1 antisera were added where indicated. The arrow indicates the Nurr1-NBRE gel shift complex. (C) Similar experiments to those described in (A) were performed, except that whole cell extracts from stimulated CD4⁺CD8⁺ (DP) and single positive (SP, CD4⁺CD8⁻ and CD4⁻CD8⁺) thymocytes were used. DP and SP thymocytes were isolated using the Coulter fluorescent-activated cell sorter. Each population was >99% pure. The total thymocytes indicated contain 90% DP T cells.

(Figure 3B). Similar results were also obtained with T-cell hybridoma extracts (data not shown). The NBRE-binding activity can be observed in both stimulated immature CD4⁺CD8⁺ and mature single positive thymocytes (Figure 3C). Addition of anti-Nur77 and anti-Nor-1 abolished most of the NBRE-binding activities in stimulated DP (CD4⁺CD8⁺) thymocytes (Figure 3C). These data indicate that Nur77 and Nor-1 constitute the majority of the NBRE-

binding activity in stimulated thymocytes and T-cell hybridomas.

To confirm the results obtained from gel-shift analysis, Western blotting was employed to probe for the expression of individual Nur77 family members in PMA/ionomycin-stimulated thymocytes. As expected, little or no Nur77 expression can be found in unstimulated thymocytes, while a high level of expression can be seen as early as 1 h

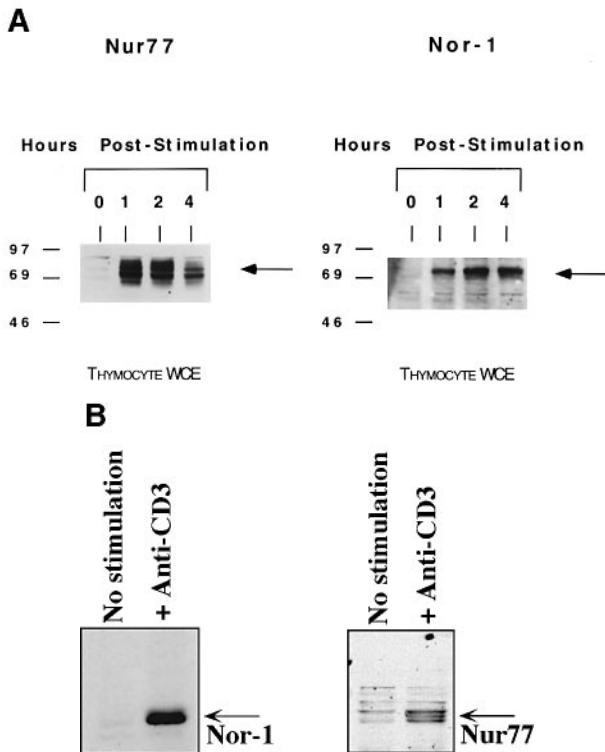


Fig. 4. Western blot analysis of the Nur77 family members in extracts from thymocytes and T-cell hybridoma (DO11.10) stimulated with a combination of PMA and ionomycin. (A) Western blot analysis of Nur77 or Nor-1 from whole cell extracts made from thymocytes (thymocyte WCE) stimulated with PMA (10 ng/ml) and ionomycin (0.25 μ M) for various lengths of time (0, 1, 2 and 4 h). Protein molecular weight standards are indicated as 97 (97 kDa), 69 (69 kDa) and 46 (46 kDa). (B) Western blot analysis of Nur77 or Nor-1 from whole cell extracts made from anti-CD3-stimulated thymocytes. Thymocytes were isolated from C57BL/6 mice that had been injected intraperitoneally with anti-CD3 antibody (1 mg) 18 h previously. This leads to apoptosis of the thymocytes, which can be observed as a reduction of total thymocyte cell number.

post-stimulation. Nur77 expression persists for at least 4 h post-stimulation, the latest time point that we have examined (Figure 4A). Nor-1 is also expressed at a high level in stimulated thymocytes (Figure 4A) and T-cell hybridomas (data not shown). Its expression can be detected easily at 1, 2 and 4 h post-stimulation. Similar results were obtained when anti-CD3 antibody was used to induce thymocyte apoptosis *in vivo* by intraperitoneal injection (Figure 4B). In contrast to Nur77/Nor-1 and consistent with the gel-shift analysis, little or no Nurr1 expression can be detected in stimulated thymocyte extracts (data not shown).

Constitutive expression of full-length Nor-1 in thymocytes results in massive apoptosis

The similar profiles of induction of Nur77 and Nor-1 suggest that they have a potentially redundant function in apoptosis. Constitutive expression of Nur77 in thymocytes leads to massive apoptosis (Calnan *et al.*, 1995; Weih *et al.*, 1996). To see if Nor-1 can also induce apoptosis in thymocytes, we generated transgenic mice constitutively expressing full-length Nor-1 protein. Transgenic mice expressing Nurr1 were also made for comparison.

To target protein expression to the thymocyte compart-

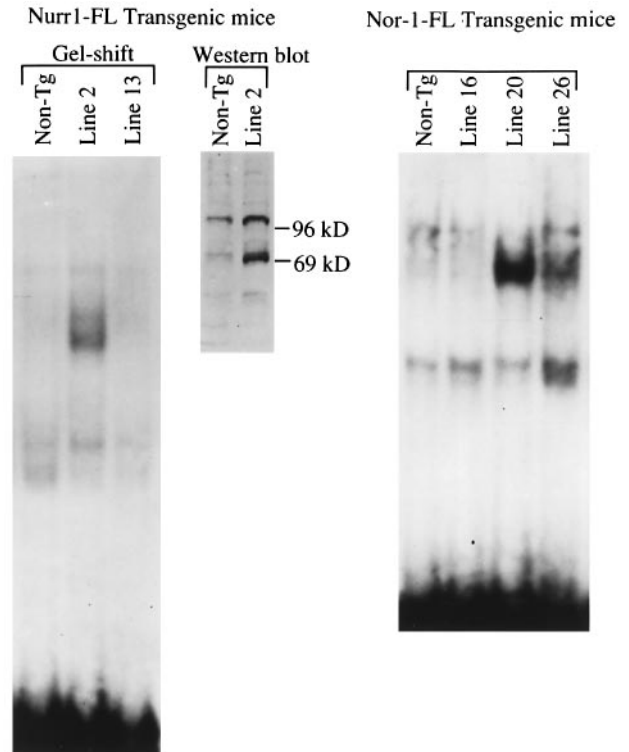


Fig. 5. Expression of the transgenic Nor-1 and Nurr1 proteins. Gel-shift analysis was performed using NBRE as a probe and Western blot analysis was performed using anti-Flag antibody (Kodak). Whole cell extracts were made from thymocytes of non-transgenic mice (Non-Tg), Nurr1-FL transgenic mice (lines 2 and 13) or Nor-1-FL transgenic mice (lines 16, 20 and 26).

ment, we employed the Ick-proximal promoter, which yields constitutive expression of Nor-1 and Nurr1. A Flag epitope tag was placed between the first and second codon of the transgenic Nurr1 construct. Several lines of transgenic Nurr1 (Nurr1-FL) and Nor-1 (Nor-1-FL) mice were generated. Transgene expression was monitored using gel-shift analysis with NBRE as a probe. Western blot analysis with the Flag antibody was also used to probe for the presence of the Nurr1 transgenic protein. Constitutive Nurr1 expression can be detected by gel-shift analysis in the thymocyte extracts of line 2 but not line 13 of the Nurr1 transgenic mice (Figure 5). This was confirmed in a Western blot analysis using the Flag-specific antibody, which detected constitutive expression of the 69 kDa transgenic Nurr1 protein (Figure 5). For the Nor-1 transgenic mice, a high level of Nor-1 protein can be seen in unstimulated thymocytes from line 20 mice (Figure 5). The specificity of the shifted complexes was confirmed by competition with unlabeled NBRE oligonucleotides. The offspring from both line 2 of the Nurr1 and line 20 of the Nor-1 transgenic mice were characterized further.

We first examined Nurr1-FL transgenic mice for any effect of the Nurr1 protein on T-cell development. Lymphocyte counts were determined in these mice and T-cell development analyzed by flow cytometry based on expression of CD3 for TCR levels and co-receptor molecules CD4 and CD8. No gross differences in the number of lymphocytes from thymus, spleen and lymph nodes can be found between the Nurr1-FL mice and their wild-type

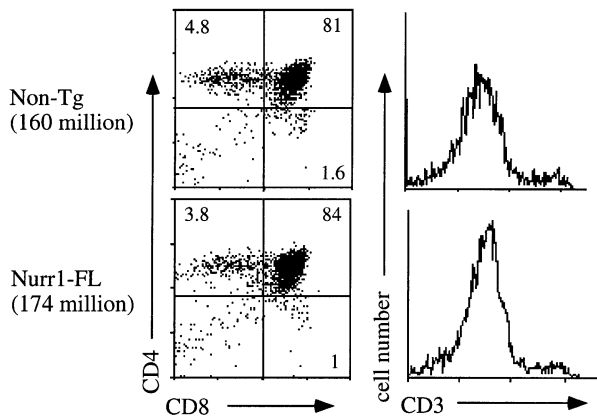


Fig. 6. Representative FACS plots of CD4, CD8 and CD3 expression on thymocytes from Nurr1-FL transgenic mice and the non-transgenic littermates (Non-Tg).

Table I. Total cellularity of the lymphoid organs from Nor-1 and Nurr1 transgenic mice

	Thymus ($\times 10^6$)	Spleen ($\times 10^6$)	Lymph nodes ($\times 10^6$)
Non-Tg littermates ($n = 4$)	128.1 \pm 33.3	42.9 \pm 10.5	5.5 \pm 4.7
Nurr1 transgenic mice ($n = 6$)	122.0 \pm 49.8	46.5 \pm 3.4	5.8 \pm 1.8
Non-Tg littermates ($n = 9$)	146.9 \pm 47.8	42.9 \pm 19.8	7.9 \pm 4.2
Nor-1 transgenic mice ($n = 10$)	10.3 \pm 5.3	31.2 \pm 8.0	2.9 \pm 1.6

littermates (Table I). Analysis of the CD4, CD8 and CD3 cell surface receptor on the thymocytes of the Nurr1-FL mice did not reveal any significant changes when compared with the non-transgenic littermates (Figure 6). FACS profiles similar to those of the wild-type mice were also obtained for the spleen and lymph nodes of these mice (data not shown). Thus, in contrast to Nur77, constitutive Nurr1 expression in thymocytes does not lead to any gross changes in T-cell development. However, as the transgenic Nurr1 protein expression is not high, we cannot rule out the possibility that a higher level of Nurr1 expression can lead to apoptosis (see Discussion).

A similar analysis was performed with the Nor-1-FL transgenic mice. In contrast to the Nurr1 transgenic mice but similarly to the Nur77-FL mice, a dramatic reduction in thymic cellularity was observed in the Nor-1 transgenic mice. In these mice, the thymocyte number is reduced 15-fold (Table I). The numbers of splenocytes and lymph nodes are also greatly reduced (Table I). The thymocyte population from these Nor-1 mice was found to contain large numbers of granular cells, measured by flow cytometry as an increase in side light scatter, suggesting an apoptotic morphology (Figure 7A).

To examine T-cell development in Nor-1-FL transgenic mice, thymocytes from these mice were examined by flow cytometry for expression of the co-receptor molecules CD4 and CD8 and for CD3. There were very few immature CD4⁺CD8⁺ or mature CD4⁺CD8⁻ and CD4⁻CD8⁺ thymocytes (Figure 7A). As thymocytes undergo apoptotic, they down-regulate CD4 and CD8, which is reflected in these mice by a relatively large population of CD4⁻CD8⁻ T cells. CD3

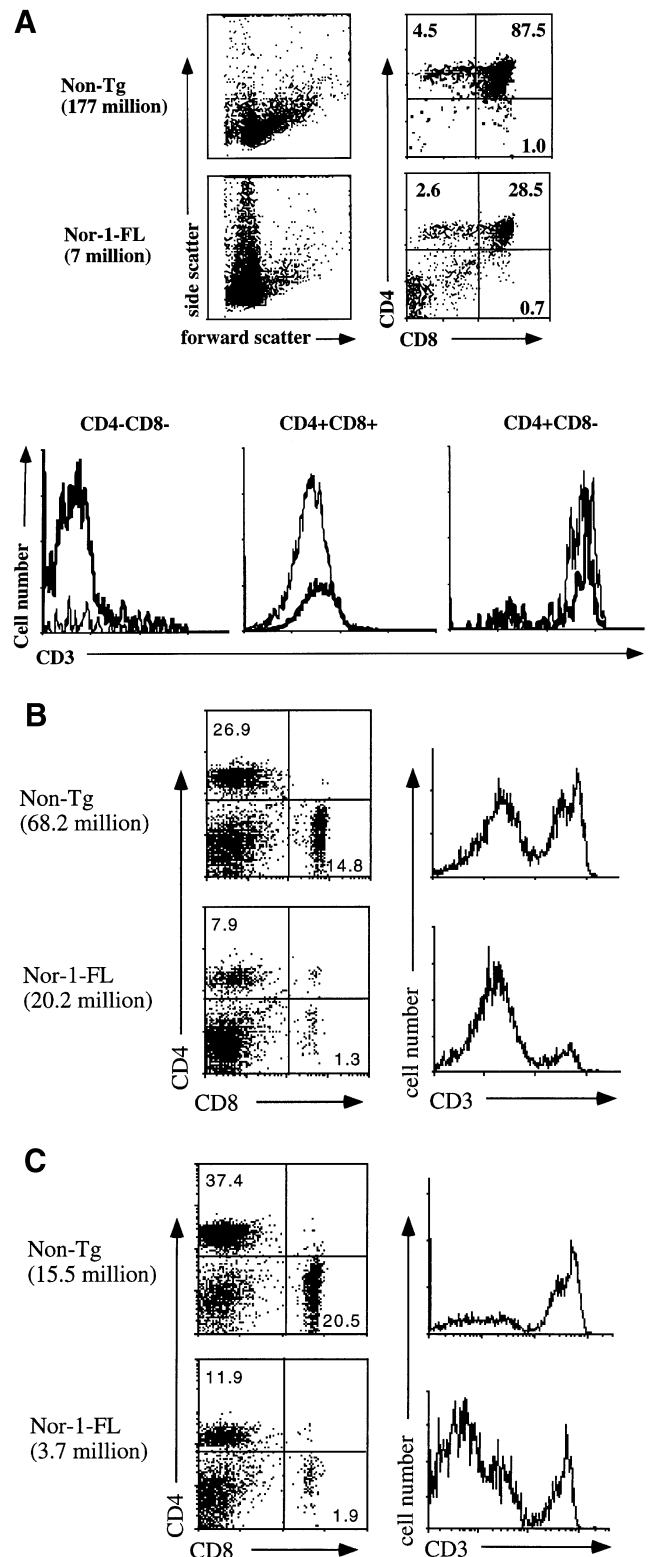


Fig. 7. Flow cytometric analysis of the Nor-1-FL transgenic mice. (A) Representative FACS plots of CD4, CD8 and CD3 expression on thymocytes from the Nor-1 transgenic mice and their wild-type littermates (Non-Tg). The thymi of the Nor-1 transgenic mice contain a large number of granular cells, as measured by an increase in side light scatter. For the CD3 plots, the bold FACS profile represents the transgenic thymocytes. (B) Representative FACS plots of CD4, CD8 and CD3 expression on splenocytes from the Nor-1 transgenic mice and their wild-type littermates (Non-Tg). (C) Representative FACS plot of CD4, CD8 and CD3 expression on lymph nodes from the Nor-1 transgenic mice and their wild-type littermates (Non-Tg).

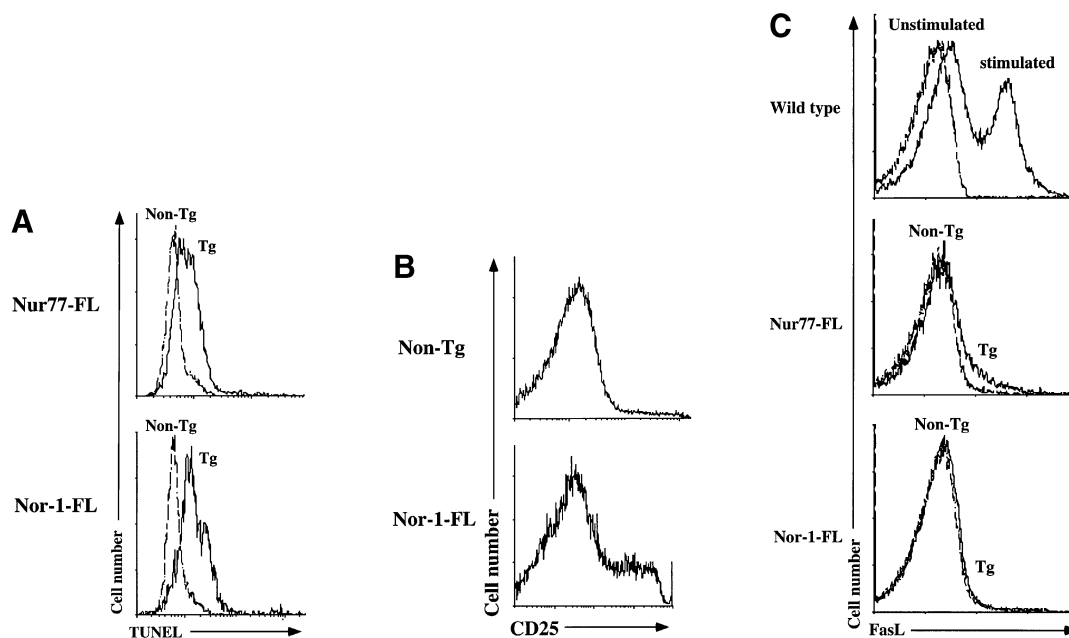


Fig. 8. TUNEL and flow cytometric analysis of the Nor-1-FL and Nur77-FL transgenic mice. (A) Representative TUNEL staining of thymocytes from a Nur77-FL mouse and its wild-type littermate, and a Nor-1-FL transgenic mouse and its non-transgenic littermate. TUNEL was performed according to the manufacturer's instructions. Briefly, thymocytes were fixed in 4% paraformaldehyde followed by permeabilization with 0.1% Triton X-100. Fragmented DNA ends were labeled with digoxigenin-11-dUTP using TdT and detected with a FITC-conjugated anti-digoxigenin antibody. (B) FACS profile of CD25 expression on thymocytes from Nur77-FL and Nor-1-FL transgenic mice as well as their wild-type littermates. (C) FACS profile of FasL expression on thymocytes from Nur77-FL and Nor-1-FL transgenic mice and their wild-type littermates. Stimulated thymocytes (PMA/ionomycin) from wild-type mice were used as a positive staining control while the unstimulated thymocytes serve as a negative control. Rabbit polyclonal antibodies recognizing mouse FasL were purchased from Santa Cruz Biotechnology Inc. Staining was done using the FasL antibodies and a secondary PE-conjugated anti-rabbit Ig antibody. The FasL staining profile of unstimulated thymocytes is identical to the staining profile obtained using only the secondary antibodies.

profiles reflect the decrease in percentages of immature $CD4^{+}8^{+}$ ($CD3^{low}$) and mature $CD4^{+}8^{-}$ and $CD4^{+}8^{+}$ thymocytes ($CD3^{high}$; Figure 7A). A population of $CD3^{-}$ cells, which represent apoptotic $CD4^{-}CD8^{-}$ T cells, is also evident in the Nor-1 transgenic mice (Figure 7A).

Lymph node cells and splenocytes from Nor-1-FL mice were also examined by flow cytometry for expression of CD4 and CD8. Although transgenic mice 4–8 weeks old had populations of both CD4 and CD8 T cells, the percentages of these cells were low compared with the percentages in non-transgenic littermate controls (spleen data in Figure 7B and lymph node data in Figure 7C). Consistent with the reduction in the number of CD4 and CD8 T cells, the percentage of CD3 cells was also found to be low (Figure 7B and C). Interestingly, a more dramatic reduction was found for the $CD8^{+}$ mature T cells than for the $CD4^{+}$ mature T cells. Similar findings can be seen in both the spleen and lymph nodes of the Nor-1 mice. These data demonstrate that Nor-1-FL mice have fewer peripheral T cells.

To demonstrate that apoptosis occurs in the Nor-1-FL thymus, we performed the TUNEL assay to detect early apoptotic cells (Gavrieli *et al.*, 1992). As shown previously, wild-type mice show few TUNEL-positive thymocytes (Figure 8A), presumably because apoptotic cells are removed rapidly by the resident macrophages (Surh and Sprent, 1994). As expected for thymocytes that undergo massive apoptosis, Nor-1-FL transgenic thymocytes showed a much higher TUNEL staining than their non-transgenic counterparts. The extent of apoptosis is similar to what has been observed in Nur77-FL transgenic thymocytes (Calnan *et al.*, 1995 and Figure 8A).

Constitutive expression of full-length Nur77 results in disappearance of anomalous autoimmune T cells from *gld/gld* FasL mutant mice

To compare the phenotype of Nor-1-FL and Nur77-FL mice further, we examined the expression pattern of CD25 and FasL on the transgenic thymocytes. An elevated level of CD25 was found previously on the Nur77-FL thymocytes (Calnan *et al.*, 1995; Weih *et al.*, 1996). We show here that a similar phenotype is also found in the Nor-1-FL thymocytes (Figure 8B).

It previously has been reported that transgenic thymocytes expressing Nur77 from the *lck*-promoter and CD2 locus control region express a high level of FasL (Weih *et al.*, 1996). To see if a similar phenotype could be observed in our Nur77-FL and Nor-1-FL transgenic mice, flow cytometric analysis using FasL antibodies was performed. Stimulated non-transgenic thymocytes were used as a positive control, while unstimulated thymocytes served as a negative control. As expected, FasL is expressed at a very high level in stimulated T cells (Figure 8C). Little FasL expression can be found in the unstimulated thymocytes (negative control). In contrast to the previous report (Weih *et al.*, 1996), however, FasL expression is not significantly different between unstimulated non-transgenic and transgenic Nur77-FL thymocytes (Figure 8C). Similarly, FasL expression is undetectable in unstimulated Nor-1-FL thymocytes (Figure 8C). This finding was confirmed further using a semi-quantitative assay reverse transcriptase-polymerase chain reaction (RT-PCR) with FasL-specific oligonucleotides. We failed to detect any significant difference in FasL mRNA levels

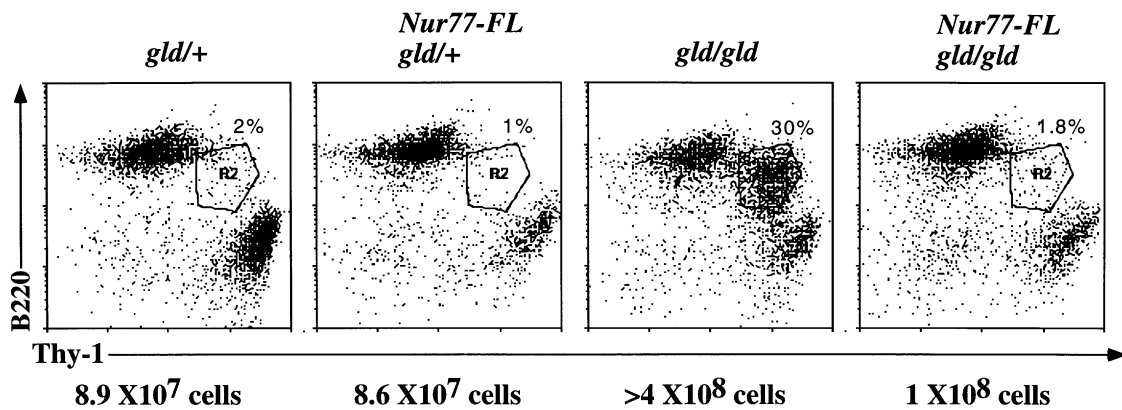


Fig. 9. Constitutive expression of Nur77 rescues the lymphoproliferative disease of the *gld/gld* strain of mice. Representative FACS plots of B220 and Thy-1 expression on the splenocytes from the various strains of mice indicated.

between thymocytes of Nur77-FL mice and their non-transgenic littermates (A.DeYoung, unpublished data).

We further investigated the possible connection between Nur77 and FasL by crossing the Nur77-FL mice to the C3H/HeJ *gld/gld* strain of mice (obtained from The Jackson Laboratory) for two generations in order to generate Nur77-FL transgenic mice in the *gld/gld* (FasL mutant) background. Typing of the FasL alleles was done with PCR using oligonucleotides which distinguish the wild-type from the *gld* mutant alleles (see Materials and methods). The mice were kept for at least 11 weeks before they were sacrificed for analysis. It is known that autoimmunity develops in *gld/gld* mice, which is accompanied by an enlargement of the lymph nodes and spleen (Cohen and Eisenberg, 1991; Giese and Davidson, 1994; Nagata and Golstein, 1995). By ~1 month of age, an anomalous population of B220⁺Thy-1⁺CD4⁻CD8⁻CD3⁺ T cells can be detected in the peripheral organs of the mutant mice (Cohen and Eisenberg, 1991; Giese and Davidson, 1994, and references therein). If FasL is the major downstream gene regulated by Nur77, introduction of Nur77 into the FasL mutant mice should have minimal effect on development of the autoimmune disease. On the other hand, if Nur77-initiated apoptosis is Fas independent, its constitutive expression may lead to abrogation of the *gld/gld* phenotype.

Analysis of several Nur77-FL *gld/gld* mice and their *gld/gld* littermates showed that constitutive expression of Nur77 eliminates the *gld/gld* phenotype (Figure 9). Enlarged lymph nodes and spleens were evident in the *gld/gld* mice, while their Nur77 transgenic littermates looked normal (data not shown). Flow cytometric analysis of splenocytes and lymph nodes was performed using B220- and Thy-1-specific antibodies. The anomalous B220⁺Thy-1⁺ population was evident in the spleen and lymph nodes of the *gld/gld* mice, while this population was absent in the Nur77-FL *gld/gld*, as well as the heterozygous *gld/+* and Nur77-FL *gld/+* mice (Figure 9 and Table II, the lymph node data are similar to the spleen data and are not shown). Consistent with the FasL staining data, we did not see any significant differences in cellular-ity or subpopulations of thymocytes in the Nur77 *gld/gld* and Nur77 *gld/+* mice (from at least five littermates, data not shown). All of these data clearly point to the conclusion that FasL is not the major downstream target of Nur77.

Table II. The percentage of B220⁺Thy-1⁺ splenic T cells from several Nur77-FL *gld/gld* and *gld/gld* mice

Age of littermates (weeks)	<i>gld/gld</i> (%)	Nur77-FL <i>gld/gld</i> (%)
24	30	1.8
22	25	3.0
11	9.1	1.8
11	9.1	1.4

Discussion

We have characterized the expression of the various Nur77 family members in thymocytes and T-cell hybridomas in response to stimulation that mimics TCR signaling. Neither the Nur77 nor Nor-1 proteins are expressed in resting T cells but are induced to a very high level when thymocytes or T-cell hybridomas are stimulated by anti-CD3 antibody or a combination of PMA/ionomycin. Nur77, on the other hand, is only transiently induced by PMA/ionomycin at a low level in T-cell hybridomas and is undetectable in PMA/ionomycin-treated thymocyte extracts. We showed that Nur77 and Nor-1 constitute most if not all of the NBRE-binding activity in stimulated thymocytes and T-cell hybridoma extracts. This NBRE-binding activity can be found in both stimulated immature (CD4⁺CD8⁺) and mature (CD4⁺CD8⁻) thymocytes. Although constitutive expression of Nur77 in thymocytes leads to apoptosis, it is not clear if expression of Nur77 in mature T cells can lead to the same outcome. Expression of Nur77 family members alone in T-cell hybridomas does not lead to apoptosis (Woronicz *et al.*, 1995).

We also showed that all three members of the Nur77 family can transactivate through the NBRE site in T cells, albeit with differing efficiencies. The dominant-negative Nur77 protein can inhibit the transactivation activity of both Nur77 and Nor-1. This would explain the difference between the dominant-negative Nur77 transgenic mice and the Nur77^{-/-} mice, since the dominant-negative protein most likely also inhibits Nor-1 protein activity *in vivo*. As the induction kinetics of Nur77 and Nor-1 are similar in T cells, the lack of a phenotype in the Nur77 mutant mice can be explained readily by the functional redundancy provided by the Nor-1 protein. The inhibitory effect of the Nur77 dominant-negative protein on negative

selection (Calnan *et al.*, 1995; Zhou *et al.*, 1996) can be explained by its interference with both Nur77 and Nor-1 proteins. The absence of any effect on the process of negative selection in the Nur77^{-/-} mice (Lee *et al.*, 1995) can simply be due to compensation by the Nor-1 protein.

While expression of the Nur77 dominant-negative protein did not result in any gross changes in T cell development (Calnan *et al.*, 1995; Zhou *et al.*, 1996), constitutive Nur77 expression leads to apoptosis. In addition to a much smaller thymus, mice with constitutive Nur77 expression (Nur77-FL) also show a smaller percentage of CD4⁺CD8⁺ thymocytes compared with their non-transgenic counterparts. TUNEL staining of tissue sections as well as of whole cells has shown extensive apoptosis in Nur77-FL transgenic thymocytes (Calnan *et al.*, 1995; Zhou *et al.*, 1996). Expression of CD44 versus CD25 in the CD4⁺CD8⁻ T-cell compartment is normal in Nur77-FL thymocytes (B.Calnan, unpublished data). Cell cycle studies of the Nur77-FL thymocytes have also indicated that most cells die in the G₀/G₁ stage (B.Calnan, unpublished data), consistent with the notion that early T-cell development is largely normal in the Nur77-FL mice.

As in the Nur77-FL thymocytes, apoptosis and up-regulation of CD25 in the CD4⁺CD8⁺ T-cell compartment were observed in thymocytes which constitutively express the Nor-1 protein. Apoptosis by Nur77 or Nor-1 is specific to the two members of the Nur77 family, as overexpression of the transcription factors Nurr1, RelA or Bcl-3 in thymocytes did not result in reduction of lymphocyte number or any other gross changes in T-cell development (Perez *et al.*, 1995; Caamano *et al.*, 1996; this study). The results with Nurr1 transgenic mice are surprising, since all the Nur77 family members can bind to and transactivate from the same DNA element. One possible explanation is the low level of Nurr1 expression in the transgenic mice. A higher level of Nurr1 protein expression might lead to apoptosis. We have, however, generated eight different transgenic founders with only one that expresses Nurr1. Furthermore, in our Nur77-FL mice, a low level of Nur77 expression is sufficient to lead to apoptosis (figure 1C in Calnan *et al.*, 1995). Indeed, transgenic Nur77 protein expression in three of our Nur77-FL founders is barely detectable by gel-shift analysis (B.Calnan, unpublished data). Another possible explanation for a lack of phenotype in the Nurr1 transgenic mice is that NBRE-binding activity alone is not sufficient for all individual Nur77 family members to initiate apoptosis. Other, as yet unknown, activities of the Nur77 and Nor-1 proteins may be necessary for the apoptotic function in thymocytes (e.g. association of Nur77 with the cell cycle machinery; Chan *et al.*, 1995).

We have examined the possible connection between Nur77 and other apoptotic pathways. Constitutive expression of Nur77 in thymocytes did not enhance the sensitivity of the cells to glucocorticoid, as similar kinetics of dexamethasone-induced cell death were observed in Nur77-FL and non-transgenic thymocytes (B.Calnan, unpublished data). Overexpression of Bcl-2 also did not rescue apoptosis significantly in the Nur77-FL transgenic mice (B.Calnan, unpublished data). FasL has been reported to be up-regulated in constitutively expressed Nur77 transgenic mice (Weih *et al.*, 1996). Mutation at FasL can, in part, rescue the apoptotic transgenic thymocytes

(Weih *et al.*, 1996). Thus, Nur77 may initiate apoptosis through the Fas pathway. Repeated flow cytometric analysis of our Nur77-FL and Nor-1-FL transgenic thymocytes, however, did not reveal any FasL expression. Furthermore, we showed that constitutive Nur77 expression leads to abrogation of the lymphoproliferative disease seen in *gld/gld* mutant mice. These data suggest that the major pathway of Nur77-initiated apoptosis is not through the Fas-FasL interaction. The discrepancy in FasL expression in the two sets of Nur77-FL mice may be due to the different levels of Nur77 protein being expressed. In our Nur77-FL mice, we used the lck-proximal promoter to drive Nur77 expression while Weih *et al.* (1996) used both the lck promoter and the CD2 locus control region. An unusually high level of Nur77 expression might indeed lead to activation of the Fas pathway. However, extensive apoptosis clearly occurs in our Nur77 and Nor-1 transgenic thymocytes in the absence of FasL expression. Thus, as in the case of apoptosis accompanying negative selection, the major apoptotic pathway initiated by constitutive Nur77 expression is Fas independent. Identification of the major downstream genes regulated by Nur77 and Nor-1 will be necessary to understand further the function of these two proteins during apoptosis.

Materials and methods

Transient transfections and luciferase assays

Jurkat cells constitutively expressing SV40 large T antigen (a generous gift from G.Crabtree) were transfected via lipofectamine (Gibco) as per the manufacturer's instructions. Two µg of DNA were transfected per 2.5 × 10⁶ cells. Cells were then harvested 36–48 h post-transfection and luciferase assays were performed after normalizing the amount of protein in the extracts. Luciferase assays were performed on an Analytical luminescence luminometer as per the manufacturer's protocol.

Antibody generation and purification

Nurr1 cDNA was cut with *Dra*II and cloned into the *Sma*I site of pGEX2T (AA1-600). The resulting plasmid was cut with *Nco*I and *Bam*HI and self-ligated to generate GST-Nurr1 (AA1-200). GST-Nor-1 was produced by PCR amplification of a 675 bp piece incorporating AA5-226. *Bam*HI sites were included in the primers and used to clone into the *Bam*HI site of pGEX1N. Both GST fusion proteins were prepared on a large scale, and emulsions were made with Freund's adjuvant via sonication (complete Freund's for the first injection and incomplete for all subsequent injections). New Zealand White rabbits were injected once every 2 weeks for 8 weeks and terminally bled 1 week after the final injection. Antibodies were then purified on a GST-Nurr1/Nor-1 affinity column with GST fusion proteins coupled to Affigel 10 (Bio-Rad) as per the manufacturer's protocol. Antisera were first pre-cleared over a GST column, then bound to a GST-Nurr1/Nor-1 column and eluted with 100 mM glycine-HCl pH 2.8.

Western blots

For thymocyte extracts, 200 µg of whole cell extract was used. For nuclear extracts, 300 µg of protein was used. Protein was run on 8% denaturing SDS-polyacrylamide gels. Proteins were then electroblotted onto nitrocellulose membranes and stained with Ponceau S to monitor for uniform loading of the gel. Blots were then blocked in 5% milk in TBST. Primary antibody was added at a concentration of 3 µg/ml. After washing, a secondary goat anti-rabbit IgG conjugated to horseradish peroxidase (Caltag) was then added at a dilution of 1:3000. Blots were developed with the Renaissance Chemiluminescence Reagent (Dupont NEN) as per the manufacturer's guidelines.

Gel-shift assays

NBRE DNA (sense and antisense strands) was annealed by mixing oligos [TCGAGTTTAAAAGGTCATGCTCAATTG (sense strand)] for 15 min at 68°C, 37°C, 25°C and 4°C successively. Oligos (which have *Sa*II 5' overhangs when annealed) were filled in with Klenow in

the presence of 50 μ Ci of 32 P-labeled dATP and dCTP (Dupont). *In vitro* transcription for Nur77 and Nurr1 was done as described (Woronicz *et al.*, 1995). For Nor-1, rat Nor-1 cDNA (a gift from N.Ohkura) in pBSK was cut with *Xba*I and self-ligated (pBSK-Nor-1 IVT) to remove an unused ATG start codon in the 5'-untranslated region. pBSK-Nor-1 IVT was then linearized with *Kpn*I and transcribed as described. *In vitro* translation was done by taking RNA and adding it to rabbit reticulocyte lysate with all amino acids (Promega) in a 50 μ l total volume. Six μ l of this reaction were used for all gel shifts. Nuclear and whole cell extracts were generated as described previously (Schreiber *et al.*, 1989; Veis *et al.*, 1993). Gel shifts with labeled NBRE as probe were performed by incubating with either *in vitro* translated proteins [in 10 mM Tris-HCl pH 8.0, 40 mM KCl, 0.5% NP-40, 6% glycerol, 1 mM dithiothreitol (DTT)] or 10 μ g of nuclear/whole cell extract (in 10 mM HEPES-KOH pH 7.9, 50 mM NaCl, 5 mM Tris-HCl pH 7.5, 15 mM EDTA, 1 mM DTT, 10% glycerol) with 75 000 c.p.m. purified probe and 1 μ g of poly(dIdC) (Pharmacia). Reactions were done on ice for 30 min, then loaded onto a 4% acrylamide gel in 0.5 \times TBE. Gels were then run for 2 h at 150 V, blotted onto Whatman paper, and dried down prior to exposure to autoradiographic film. For blocking experiments, proteins were incubated with antibody (1 μ l of whole serum or 1 μ g of purified antibody) on ice for 1 h. Probe was then added and the reaction was performed as described (Woronicz *et al.*, 1995).

Generation of transgenic mice

For the Nurr1 Flag Tag transgenic construct, four primers were used in a PCR reaction using the pBSK-Nurr1 plasmid as a template: Nurr1A-CAATTAACCCCTACTAAAGGGAAC (sense), Nurr1B-TTTATCGT-CATCGTCTTTGTAGTCCATGGCTTCAGCAGAGTTATTGGA (antisense), Nurr1C-GACTACAAAGACGATGACGATAAACCTTGTGTT-CAGGCGCAGTATGG (sense), and Nurr1D-CTGTCCGGACAGGGG-CATTTC (antisense). The PCR product was then cut with *Xba*I and *Bsa*HI and cloned into *Xba*I-*Bsa*HI-cut Nurr1 cDNA in pBSK. Proper insertion and the sequence of PCR fragment were verified via sequence analysis. The Flag-Nurr1 construct was then cut with *Xba*I-*Xho*I, filled in with Klenow, and cloned into a filled-in *Bam*HI site of a vector containing the lck-proximal promoter (p1017). The transgene was then liberated by cutting with *Not*I and subsequently purified prior to injection.

The Nor-1 transgene was constructed by cutting the full-length Nor-1 cDNA in pBSK with *Xba*I-*Eco*RI, filling in with Klenow, and cloning into the filled-in *Bam*HI site of p1017 (Chaffin *et al.*, 1990). The transgene was liberated by cutting with *Spe*I, purified, and injected into mouse embryos. Founders for Nurr1 were typed by cutting tail DNA with *Eco*RI and performing Southern blot analysis. For Nor-1, tail DNA was cut with *Pst*I. For Nurr1 mice, F1 mice were typed by PCR using a specific primer for the Flag tag and Nurr1D. The following oligonucleotides were used to type the Nur77-FL mice: pEVRFN-term (GGGGGATCTTGGTGGCGTG) and ceder-2 (GCGGATCCGGGG-AGGCATCTGGAGGCTG). For the Nor-1-FL mice, the following oligonucleotides were used: pplck2 (TGGTGCTTGAGGGCTCAGAGG) and Nor1AA1rev (GCGCGCTGAATGGTGGGGCT). To distinguish the *gld/gld* strain of mice from *gld*⁺ mice, we used the following oligonucleotides: (i) for the FasL wild-type sequence, *gld*^{5'} (GTCCATCCCTCTGG-AATGG) and *gld*^{3'}wt (GCTTATACAAGCCGAAAAA); (ii) for the FasL *gld* mutation, *gld*^{5'} and *gld*^{3'} (GCTTATACAAGCCGAAAAA) (Takahashi *et al.*, 1994). PCR was performed at 57°C with 40 ng of each oligonucleotide to allow specific amplification of the mutant (using *gld*^{5'} and *gld*^{3'}) or wild-type (using *gld*^{5'} and *gld*^{3'}wt) alleles. As controls, we showed that under the above PCR condition, the *gld*^{5'} and *gld*^{3'} oligonucleotides amplified a fragment of the expected size from the DNAs of *gld/gld* but not from the wild-type strain of mice. Conversely, PCR reactions using the *gld*^{5'} and *gld*^{3'}wt oligonucleotides specifically gave rise to the expected FasL fragment from the wild-type but not the *gld/gld* DNAs. Both sets of primers amplified the FasL fragment from DNA of *gld*⁺ strain of mice.

Cell counts and flow cytometry

Thymus, spleen and lymph node were isolated from mice and placed in RPMI supplemented with 5% fetal calf serum (FCS). Cell suspensions were generated, strained and washed twice in RPMI. Cells were counted, then stained with appropriate antibodies (Caltag or Pharmingen) in 2% FCS and 1 \times phosphate-buffered saline (PBS). (Antibodies used were: anti-CD3 biotin, anti-CD4-PE, anti-CD8-FITC, anti-B220-PE, anti-Thy-1-tricolor, anti-CD25.) The FasL antibodies were purchased from Santa Cruz Biotechnology Inc. Cells were then washed twice in 2% FCS and 1 \times PBS, resuspended in 1 \times PBS, and run on a Coulter EPICS XL flow cytometer.

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