*Journal of Molecular Neuroscience*
Copyright © 1996 Humana Press Inc.
All rights of any nature whatsoever reserved.
ISSN0895-8696/95/6:249–255/$6.75

# Three Related Brain Nuclear Receptors, NGFI-B, Nurr1, and NOR-1, as Transcriptional Activators

***Ragnhild E. Paulsen,*[*,1,2] Kjersti Granås,[1] Helge Johnsen,[1] Veslemøy Rolseth,[1] and Sigrun Sterri[1]***

Received December 4, 1995; Accepted December 29, 1995

*[1]Norwegian Defence Research Establishment, Division for Environmental Toxicology, N-2007 Kjeller, Norway; and [2]Institute of Pharmacy, University of Oslo, Biotechnology Centre of Oslo, N-0316 Oslo, Norway*

## Abstract

Three related orphan nuclear receptors that are expressed in the brain, NGFI-B, Nurr1, and NOR-1, were studied to compare their function as transcriptional activators. NGFI-B was able to activate (in the absence of added hormone) in CV1 cells both an NGFI-B-responsive luciferase reporter gene (containing eight copies of a response element for NGFI-B upstream of a basal prolactin promoter driving the luciferase gene, $NBRE_8$-LUC), a similar thyroid hormone-receptor-responsive reporter gene ($TRE_3$-LUC), and a reporter gene with an authentic promoter from a *Xenopus* vitellogenin gene containing two binding sites for the estrogen receptor (vit-LUC). NGFI-B activated $NBRE_8$-LUC and $TRE_3$-LUC (but not the vit-LUC) with an amino-terminal activation domain. Nurr1 was less promiscuous as a transcriptional activator, activating the $NBRE_8$-LUC better than NGFI-B, but less than NGFI-B at the other reporter genes. NOR-1 activated only the $NBRE_8$-LUC reporter gene. These results indicate that closely related nuclear receptors may differentiate between response elements or promoters and that different activation mechanisms exist depending on the promoter. This may contribute to regulation of specificity of target gene expression in the brain.

**Index Entries:** Gene regulation; NGFI-B; NOR-1; Nurr1; transcription factor.

**Abbreviations:** ERE, estrogen receptor response element; GRE, glucocorticoid receptor response element; NGFI-B, nerve growth factor-induced clone B; NBRE, NGFI-B response element; NOR-1, neuron-derived orphan receptor; Nurr1, Nur-related factor 1; TRE, thyroid hormone receptor response element.

*Author to whom all correspondence and reprint requests should be addressed.

## Introduction

NGFI-B is an immediate early gene of the steroid-thyroid receptor superfamily, also called nuclear receptors, whose members encode ligand-dependent transcription factors (Evans, 1988). NGFI-B was originally identified by virtue of its rapid activation by NGF in rat PC12 pheochromocytoma cells (Milbrandt, 1988). It is also called Nur77 and is induced by serum in mouse fibroblasts (Hazel et al., 1988). NGFI-B is called an orphan member of the nuclear receptor superfamily because it shows a strong sequence homology to other members, but a specific ligand for NGFI-B has not been identified (Wilson et al., 1991). The recent identification of two new brain-specific nuclear receptor transcription factors, Nurr1 (mouse; Law et al., 1992) and NOR-1 (rat; Ohkura et al., 1994), with amino acid sequence closely related to NGFI-B suggests that these proteins constitute a subfamily within the nuclear receptor superfamily. Unlike NGFI-B, which is expressed in most tissues (Watson and Milbrandt, 1990), Nurr1 is almost exclusively localized to the brain (Law et al., 1992). NOR-1 was isolated from cultured forebrain neurons undergoing apoptosis (Ohkura et al., 1994).

Like many immediate early genes, the transcription of the NGFI-B gene is activated transiently, rapidly, and independent of protein synthesis in a variety of cell types by multiple stimuli, including growth factors, seizure induction, calcium ionophores, and phorbol esters (Watson and Milbrandt, 1989; Hazel et al., 1991). Nurr1 is also induced by external stimuli, but with a differential selectivity compared to NGFI-B (Law et al., 1992). NOR-1 induction has not yet been characterized.

The presence of three similar orphan nuclear receptors in the brain with more restricted distribution elsewhere in the body raises the question of whether they serve specific roles as transcriptional activators in the brain or whether there is a redundancy in their expression. Little is known about which target genes are activated and the mechanisms of regulation of the target genes that are activated by these three transcription factors.

The aim of the present article is to address the question of whether there are specific differences among NGFI-B, Nurr 1, and NOR-1 as transcriptional activators. To this aim we have investigated the capability of NGFI-B, Nurr1, and NOR-1 to activate three different reporter genes in cultured fibroblasts (CV1 cells). In addition, we have studied the domains in the NGFI-B protein involved in the transactivation process.

## Materials and Methods

### *Recombinant Plasmids*

The expression vector for NGFI-B has been described earlier (Wilson et al., 1991; Paulsen et al., 1992). The aminoterminal deletion mutant of NGFI-B, BΔ25–195, was produced by cleaving with specific restriction enzymes within the cDNA (Milbrandt, 1988) and inserting a *Bam*HI linker to maintain the correct reading frame. Mutant BΔ360–597 is NGFI-B truncated at amino acid residue 360 and was produced by cleaving with *Nae*I and inserting an oligonucleotide providing the remaining codons and ending with a stop codon. Both these mutants have previously been described (Paulsen et al., 1992). Nurr1 cDNA was a gift from O. M. Conneely, Baylor College of Medicine, Texas, and NOR-1 was a gift from N. Ohkura, Terumo Research and Development Center, Kanagawa, Japan. In NOR-1, the first ATG codon was deleted by *Xba*I digestion and self-ligation as recommended by N. Ohkura. Both Nurr1 and NOR-1 were inserted into the mammalian expression vector pCMV. Expression vectors for estrogen receptor (ER), glucocorticoid receptor (GR), and thyroid hormone receptor (TR) were kindly provided by J. Milbrandt, Washington University, St. Louis, MO. Expressor plasmids encoding chimeric proteins of the DNA bind-

ing region (amino acids 1–147) of the yeast transcription factor GAL4, fused to select regions of NGFI-B, were produced from pSG424, which encodes the first 147 amino acids of GAL4 (Sadowski and Ptashne, 1989), moved into pCMV as a *Hin*dIII-*Xba*I fragment (plasmid pJDM 882, kindly provided by J. Milbrandt, Washington University) with insertion of restriction fragments from NGFI-B. The constructs were: GAL4(1–147)-B(33–196), GAL4 (1–147)-B(33–71), GAL4(1–147)-B(136–196), and GAL4(1–147)-B(347–597). The constructs were verified by seqencing (Sanger et al., 1977). Subsequently, using PRC cloning, a hemagglutinin epitope (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-Ser) for potential immunoprecipitation and a stop codon were introduced at the end of the fusion constructs.

### *Tissue Culture, Transient Transfection, and Luciferase Assay*

CV1 cells were grown in Dulbecco's modified Eagles' medium supplemented with 10% fetal calf serum. A calcium phosphate precipitation procedure was used for transient transfections. The reporter gene plasmids were $NBRE_8$-LUC or $TRE_3$-LUC (containing eight and three copies of the response elements for NGFI-B [5'-AAAAGGTCA-3'] or thyroid hormone receptor [5'-AGGTCATGACCT-3'], respectively, upstream of the minimal prolactin promoter driving the bacterial luciferase gene) (Wilson et al., 1991), vit-LUC (an authentic promoter from a *Xenopus* vitellogenin gene containing two response elements for the estrogen receptor fused to the luciferase gene), or GAL-4-LUC (pJDM914, a reporter gene for GAL4 fusions containing a binding site for the GAL4 DNA binding domain), all kindly provided by J. Milbrandt. Two micrograms of the reporter gene plasmid, 1 μg rsvCAT (as in Paulsen et al., 1992) as an internal control, and 1–4 μg of the various expression constructs were mixed together with carrier DNA (nonrecombinant pCMV) to a total DNA concentration of 10 μg/10-cm plate of cells. After the addition of DNA, the cells were grown in the same medium for 48 h and harvested in 0.1% SDS in 50 m*M* Tris-MES, pH 7.8, with 1 m*M* DTT for luciferase assay (De Wet et al., 1987) and chloramphenicol acetyltransferase (CAT) assay (Seed and Shean, 1988). To normalize for transfection efficiency, luciferase activities were corrected for CAT activity. Each construct was tested multiple times with two or more different plasmid preparations.

## Results

NGFI-B, Nurr1, and NOR-1 are transcriptionally active members of the nuclear receptor family. To identify potentially different functions of the three activators, we wanted to look for differences in the way that they activate different reporter genes. CV1 cells were used for these studies because they do not express any detectable levels of these proteins under the culturing conditions and it is therefore possible to study the three transcription factors independently.

NGFI-B, Nurr1, or NOR-1 expressor plasmids were transfected together with an NGFI-B-responsive luciferase reporter gene containing eight copies of an NGFI-B response element ($NBRE_8$-LUC), a TR-responsive reporter gene containing three copies of a TR-responsive element ($TRE_3$-LUC), or a reporter gene containing a naturally occurring vitellogenin promoter containing two ER-responsive elements (vit-LUC) (Fig. 1). The transcriptional activity on the NBRE as measured by the level of the luciferase gene product was highest for Nurr1, followed by NGFI-B and NOR-1. The transcriptional activity of NGFI-B on TRE and ERE (contained in the vitellogenin promoter), however, was larger (about twofold) than of Nurr1, and NOR-1 was transcriptionally inactive on TRE and ERE. This indicates that NGFI-B, Nurr1, and NOR-1 have different selectivities for the promoter of these reporter genes. For comparison, NGFI-B activated TRE and ERE

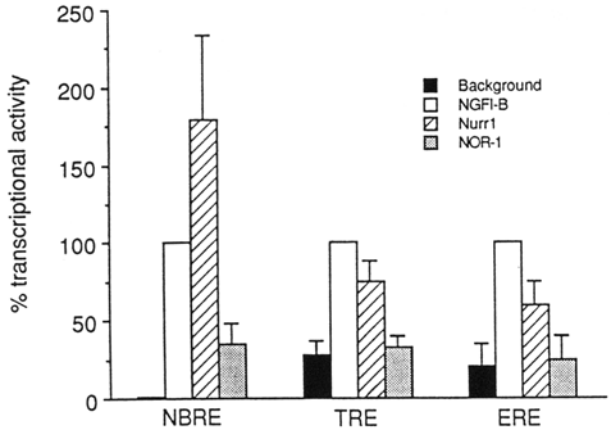


Fig. 1. NGFI-B is less specific as a transcriptional activator than Nurr1 and NOR-1. CV1 cells were transfected with 2 μg reporter gene ($NBRE_8$-LUC, labeled NBRE; $TRE_3$-LUC, labeled TRE; or vit-LUC, labeled ERE) together with 4 μg of NGFI-B, Nurr1, or NOR-1, 1 μg rsvCAT, and 3 μg pCMV, and harvested after 48 h. Luciferase and CAT activities were measured as described in Materials and Methods. Data are expressed as a percentage of NGFI-B, mean ± SEM from five experiments, using CAT as an internal standard.

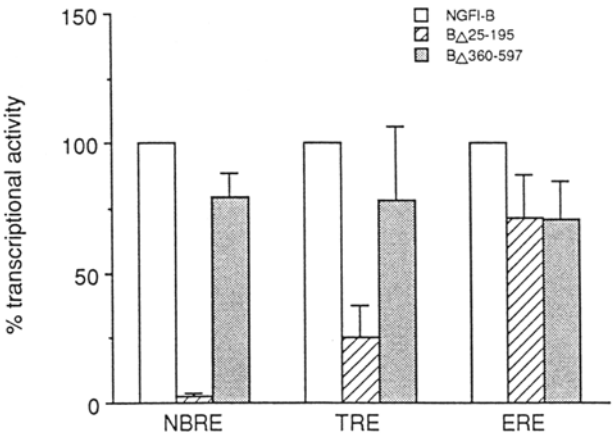


Fig. 2. NGFI-B uses the same activation domain when activating from NBRE and TRE. CV1 cells were transfected with 2 μg reporter gene ($NBRE_8$-LUC, labeled NBRE; $TRE_3$-LUC, labeled TRE; or vit-LUC, labeled ERE) together with 4 μg of NGFI-B, BΔ25-195, or BΔ360-597, 1 μg rsvCAT, and 3 μg pCMV, and harvested after 48 h. Luciferase and CAT activities were measured as described in Materials and Methods. Data are expressed as percent of NGFI-B with the background subtracted, mean ± SEM from 3–8 experiments, using CAT as an internal standard.

with 7 and 14% of the corresponding activity obtained with TR and ER in the presence of their hormones (triiodothyronine and estradiol, respectively, not shown). Neither NGFI-B nor Nurr1 activated a similar GR-responsive reporter gene (GRE-LUC), which was activated by GR in the presence of dexamethasone (not shown).

Since NGFI-B was able to activate several reporter genes, we wanted to investigate whether the same activating mechanism was involved for the different reporter genes. Because transcriptional activation domains are located within the amino terminus or carboxyl terminus of different nuclear receptors, we tested both an amino-terminal deletion mutant (BΔ25-195) and a carboxyl-terminal deletion mutant (BΔ360-597) and compared these to wild-type NGFI-B (Fig. 2). A substantial reduction in transcriptional activity was observed on NBRE and TRE with BΔ25-195, and no such reduction was observed with BΔ360-597, indicating that the amino-terminal activating domain may be used when activating from NBRE and TRE. With vit-LUC (ERE) there was only a small reduction in transcriptional activity when deleting either the amino-terminal amino acids 25–195 or the carboxyl terminus.

To identify domains within the NGFI-B sequence that are responsible for the transactivation, we produced fusion constructs of the yeast transcription factor GAL4-DNA binding domain and select regions of the NGFI-B protein, and investigated their ability to activate a GAL4-responsive reporter gene (Fig. 3). The GAL4 DNA-binding domain alone did not activate the reporter gene. Fusion constructs containing amino acids 33–196, 33–71, or 136–196 from the amino terminus activated the reporter gene, indicating that the amino-termi-

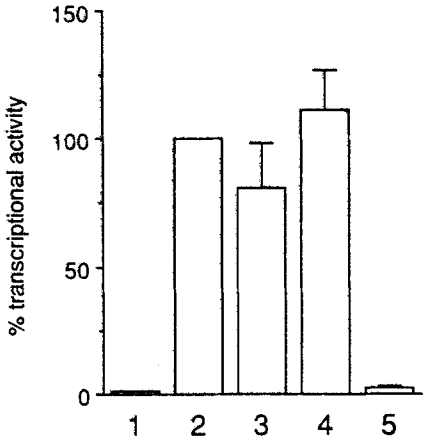


Fig. 3. Verifying activation domains in NGFI-B. CV1 cells were transfected with 2 μg reporter gene (GAL-4-LUC) together with 1 μg of the expressor plasmids GAL4(1–147) (bar 1), GAL4(1–147)-B(33–196)-HA (bar 2), GAL4(1–147)-B(33–71)-HA (bar 3), GAL4(1–147)-B(136–196)-HA (bar 4), or GAL4 (1–147)B(347–597) (bar 5), 1 μg rsvCAT, and 6 μg pCMV, and harvested after 48 h. Luciferase and CAT activities were measured as described in Materials and Methods. Data are expressed as a percentage of GAL4(1–147)-B(33–196)-HA, mean ± SEM from five experiments, using CAT as an internal standard.

nal activation domain 33–196 consists of at least two independent activation domains. The carboxyl terminus only showed a minor increase above background when activating the reporter gene as a fusion construct with the GAL4 DNA binding domain.

## Discussion

In the present article it was shown that transcription factors NGFI-B, Nurr1, and NOR-1 have different selectivities toward different artificial reporter genes and that different activating mechanisms exist for the transactivation from different promoters. NGFI-B, Nurr1, and NOR-1 are all expressed in the brain at different levels throughout development and in response to specific stimuli (Watson and Milbrandt, 1990; Law et al., 1992; Ohkura et al., 1994). Functional activity has been described in cultured neurons and astrocytes (Ciani and Paulsen, 1995). Of special interest is that NGFI-B has been shown to be necessary for apoptosis in T-cell hybridomas (Liu et al., 1994; Woronicz et al., 1994), although no phenotype was observed in gene knockout mice (Lee et al., 1995). It has been suggested that NGFI-B involved in apoptosis is regulated differently than NGFI-B involved in other processes (Winoto, 1994). Quite interesting, also, is that NOR-1 was isolated from forebrain neurons undergoing apoptosis (Ohkura et al., 1994). It is therefore of great interest to elucidate possible differences among the NGFI-B, Nurr1, and NOR-1 as transcriptional activators and to investigate if different activation mechanisms are involved when turning on genes with different promoters. Therefore, we have studied three reporter genes with different promoters. NGFI-B, Nurr1, and NOR-1 were all able to activate the reporter gene containing response elements described to be a specific binding site for NGFI-B, the NBRE, which contains a TRE/ERE half site and three adjacent A-T base pairs (Wilson et al., 1991). The basis for this recognition is the presence of Glu-Gly-x-x-Gly in the P-box in the first zinc finger, which recognizes the TRE/ERE half site (for review, *see* Freedman and Luisi, 1993) and the A box downstream of the second zinc finger, which recognizes the adjacent A-T base pairs (Wilson et al., 1992). NGFI-B and Nurr1, but not NOR-1, were also able to activate from TRE and ERE, despite the requirement of the adjacent A nucleotides in the half-site high-affinity binding site (Wilson et al., 1991), and the absence of binding of NGFI-B to single ERE/TRE binding sites in vitro (Wilson et al., 1991). This may indicate that inside cells, lower affinity sites also may promote binding and transcriptional activity. It could be that the presence of several low-affinity binding sites together provides the necessary stability of the protein–DNA complex. Different selectivity of NGFI-B, Nurr1, and NOR-1 toward these low-affinity sites may

provide a mechanism of selective target gene activation. Neither of the proteins activated a GRE-LUC, which contains a different half site (5'-AGAACA-3') that is recognized by a different P-box (Gly-Ser-x-x-Val). Binding to TRE/ERE but not GRE is in agreement with the specific residues determining half-site specificity and indicates that activation from NBRE, TRE, and ERE is a specific action.

Many nuclear receptors contain activation domains in the amino terminus and carboxyl terminus of the protein (Beato, 1989). NGFI-B activated from NBRE, TRE, and ERE. It was interesting to investigate if the same activating mechanism was involved when activating from the three different reporter genes. It seems as if the amino terminus is important when activating from NBRE and TRE, but not from the naturally occurring promoter from a vitellogenin gene containing EREs. A similar observation was made for the estrogen receptor, in which the amino terminus was required for full activation from some estrogen-responsive genes (human pS2 gene promoter), but not others (e.g., *Xenopus* vitellogenin estrogen-responsive promoter) (Kumar et al., 1987).

We have shown that NGFI-B contains two independent activation domains in the amino terminus but only a very weak activity in the carboxyl terminus, when expressed as fusion proteins with the GAL4 DNA binding domain. This is in agreement with a deletion analysis of the wild-type NGFI-B, where it was shown that a deletion of amino acids 16–71 or 137–195 both showed a significant reduction in the transcriptional activity, whereas a deletion of the carboxyl-terminal amino acids 360–597 did not affect its activity (Paulsen et al., 1992). One activation domain is acidic (amino acids 33–71 of the present study), and the other contains proline-rich stretches (amino acids 136–196 of the present study), two important subtypes of activation domains (Mitchell and Tjian, 1989). The presence of more than one independent activation domain in the protein provides a potential mechanism for specificity of target gene regulation. The nature of the activation domains of Nurr1 and NOR-1 remains to be established.

In conclusion, NGFI-B contains more than one activation domain, and NGFI-B, Nurr1, and NOR-1 activate different reporter genes with different efficiencies, both with potential implication for their regulation of target genes in the brain in vivo.

## Acknowledgment

The authors wish to thank J. Milbrandt and T. Fahrner (Washington University, St. Louis, MO) for fruitful discussions, R. Skibakk (NDRE) for expert technical assistance, and E. Biering (NDRE) for expert sequencing.

## References

Beato M. (1989) Gene regulation by steroid hormones. *Cell* **56,** 335–344.

Ciani E. and Paulsen R. E. (1995) Activation of a reporter gene responsive to NGFI-B in cultured neurons and astrocytes. *J. Mol. Neurosci.* **6,** 131–139.

De Wet J. R., Wood K. V., De Luca M., Helinski D. R., and Subramani S. (1987) Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* **7,** 725–737.

Evans R. M. (1988) The steroid and thyroid hormone receptor superfamily. *Science* **67,** 889–895.

Freedman L. P. and Luisi B. F. (1993) On the mechanism of DNA binding by nuclear hormone receptors: a structural and functional perspective. *J. Cell. Biochem.* **51,** 140–150.

Hazel T. G., Nathans D., and Lau L. F. (1988) A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. *Proc. Natl. Acad. Sci. USA* **85,** 8444–8448.

Hazel T. G., Misra R., Davis I. J., Greenberg M. E., and Lau L. F. (1991) Nur 77 is differentially modified in PC12 cells upon membrane depolarization and growth factor treatment. *Mol. Cell. Biol.* **11,** 3239–3246.

Kumar V., Green S., Stack G., Berry M., Jin J.-R., and Chambon P. (1987) Functional domains of the human estrogen receptor. *Cell* **51,** 941–951.

Law S. W., Conneely O. M., DeMayo F. J., and O'Malley B. W. (1992) Identification of a new brain-specific transcription factor, Nurr1. *Mol. Endocrinol.* **6,** 2129–2135.

Lee S. L., Wesselschmidt R. L., Linette G. P., Kanagawa O., Russell J. H., and Milbrandt J. (1995) Unimpaired thymic and peripheral T cell death in mice lacking the nuclear receptor NGFI-B (Nur77). *Science* **269,** 532–535.

Liu Z.-G., Smith S. W., McLaughlin K. A., Schwartz L. M., and Osborne B. A. (1994) Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene *nur77*. *Nature* **367,** 281–284.

Milbrandt J. (1988) Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. *Neuron* **1,** 183–188.

Mitchell P. J. and Tjian R. (1989) Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245,** 371–378.

Ohkura N., Hijikuro M., Yamamoto A., and Miki K. (1994) Molecular cloning of a novel thyroid/steroid receptor superfamily gene from cultured rat neuronal cells. *Biochem. Biophys. Res. Commun.* **205,** 1959–1965.

Paulsen R. E., Weaver C. A., Fahrner T. J., and Milbrandt J. (1992) Domains regulating transcriptional activity of the inducible orphan receptor NGFI-B. *J. Biol. Chem.* **267,** 16,491–16,496.

Sadowski I. and Ptashne M. (1989) A vector for expressing GAL-4(1-147) fusions in mammalian cells. *Nucleic Acids Res.* **17,** 7539.

Sanger F., Nicklen S., and Coulson A. R. (1977) DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.

Seed B. and Shean J. Y. (1988) A simple phase-extraction assay for chloramphenicol acyltransferase activity. *Gene* **69,** 271–277.

Watson M. A. and Milbrandt J. (1989) The NGFI-B gene, a transcriptionally inducible member of the steroid receptor gene superfamily: genomic structure and expression in rat brain after seizure induction. *Mol. Cell. Biol.* **9,** 4213–4219.

Watson M. A. and Milbrandt J. (1990) Expression of the nerve growth factor-regulated NGFI-A and NGFI-B genes in the developing rat. *Development* **110,** 173–183.

Wilson T. E., Fahrner T. J., Johnston M., and Milbrandt J. (1991) Identification of the DNA binding site for NGFI-B by genetic selection in yeast. *Science* **252,** 1296–1300.

Wilson T. E., Paulsen R. E., Padgett K. A., and Milbrandt J. (1992) Participation of non-zinc finger residues in DNA binding by two nuclear orphan receptors. *Science* **256,** 107–110.

Winoto A. (1994) Molecular characterization of the Nur77 orphan steroid receptor in apoptosis. *Int. Arch. Allergy Immunol.* **105,** 344–346.

Woronicz J. D., Calnan B., Ngo V., and Winoto A. (1994) Requirement for the orphan steroid receptor Nur77 in apoptosis of T-cell hybridomas. *Nature* **367,** 277–281.