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Differential transcription of the orphan receptor RORβ in nuclear extracts derived from Neuro2A and HeLa cells

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ABSTRACT

An important model system for studying the process leading to productive transcription is provided by the superfamily of nuclear receptors, which are for the most part ligand-controlled transcription factors. Over the past years several 'orphan' nuclear receptors have been isolated for which no ligand has yet been identified. Very little is known about how these 'orphan' receptors regulate transcription. In this study we have analysed the biochemical and transcriptional properties of the neuronally expressed orphan nuclear receptor RORβ (NR1F2) and compared them with the retinoic acid receptor heterodimer RXRα–RARα (NR2B1–NR1B1) and Gal– VP16 *in vitro*. Although RORβ binds to its DNA-binding sites with comparatively low affinity, it efficiently directs transcription in nuclear extracts derived from a neuronal cell line, Neuro2A, but not in nuclear extracts from non-neuronal HeLa cells. In contrast, RXRα–RARα and the acidic transcription factor Gal–VP16 support transcription in Neuro2A and HeLa nuclear extracts equally efficiently. These observations point to a different (co)factor requirement for transactivation by members of the NR1 subfamily of nuclear receptors.

INTRODUCTION

The spatio-temporal regulation of gene expression is one of the key issues in differentiation and development. A central role in the control of gene expression is played by transcription factors that bind to specific enhancer/promoter elements and activate or repress transcription of a specific gene. The complex mechanism of transcriptional activation permits tight control at multiple levels, such as binding of the transcription factor to its response element, recruitment of co-activator/co-repressor complexes and the basal transcription machinery and facilitating reinitiation of transcription.

Among many model systems the nuclear receptors of the thyroid/steroid superfamily have been of particular importance in the continuing dissection of the mechanisms responsible for control of transcription. The nuclear receptor superfamily now comprises more than 50 distinct members which have been grouped into six different subfamilies based on sequence similarities in the two well-conserved regions of this family, the DNA-binding and the ligand-binding domains (1).

The 'classical' nuclear receptor acts as a ligand-controlled transcription factor which responds either to steroid hormones, small lipophilic molecules or vitamins. The last few years have witnessed major breakthroughs in understanding the molecular mechanisms of transcription signal transmission by these receptors (for reviews see 2,3 and references therein). In addition to these 'classical' receptors, a rather large number of proteins have been identified that share the overall protein structure of nuclear receptor family members but for which no ligand has (yet) been found. These receptors are commonly referred to as 'orphan' receptors (4).

Some orphan receptors have DNA-binding and dimerisation properties very similar to those of 'classical' receptors, including their ability to bind as homodimers or RXR heterodimers to half-sites arranged either as a palindrome or direct repeats. However, a subgroup of orphan receptors exists that bind as monomers to so-called extended half-sites. Nucleotides 5′ of the conserved receptor half-site (AGGTCA) are contacted by these orphan receptors increasing DNA binding affinity and providing specificity in the recognition of monomeric response elements (5). An intriguing question is how the transcriptional activity of orphan receptors is regulated in the absence of a ligand and of a heterodimerisation partner. It is possible that the transcriptional activity of orphan receptors is controlled by protein modifications. Alternatively, these receptors may display a constitutive ability to bind either co-repressor or co-activator complexes.

A good model system for studying transcriptional control by orphan receptors that bind to DNA as monomers is the subgroup of the ROR (NR1F) (also referred to as RZR) and the Rev-erb (NR1D) receptors. In transient transfections members of the ROR subgroup appear to act as constitutive activators, whereas members of the Rev-erb subgroup act as constitutive repressors.

Two members of the ROR subgroup, RORα (NR1F1) (6,7) and RORβ (NR1F2) (8), are highly related but display very different expression patterns. While RORα is ubiquitously expressed, RORβ expression is restricted to neuronal cells, in particular to areas in the central nervous system that are

involved in the processing of sensory information (spinal cord, thalamus and sensory cerebellar cortices) and the major areas of circadian rhythm regulation (retina, pineal gland and suprachiasmatic nuclei) (9). Despite the high degree of amino acid similarity, we recently demonstrated that these two receptors display different DNA-binding behaviors (10), suggesting that specific transcriptional regulation mechanisms may be in place for these transcription factors.

To unravel the mechanism underlying regulation of transcription by monomeric binding orphan receptors, we expressed RORβ using the vaccinia virus system and compared its DNA-binding and transcriptional properties with those of the RXRα–RARα (NR2B1–NR1B1) heterodimer and the synthetic acidic activator Gal–VP16 *in vitro*. In this report we show that although RORβ has a weak affinity for its response elements compared with the RXRα–RARα heterodimer, it potently drives transcription in Neuro2A but not in HeLa nuclear extracts. RXRα–RARα and Gal–VP16 are able to efficiently activate transcription in both Neuro2A and HeLa nuclear extracts.

MATERIALS AND METHODS

Recombinant vaccinia viruses

Recombinant vaccinia viruses expressing His_6–RORβ and RXRα–His_6–RARα were prepared according to the protocol described (10,11). The expression vector for RORβ contained the $RORβ_1$ cDNA (8) in pMS56 (12). The amino acids -MSHHHH-HHGEF precede the second amino acid of the RORβ sequence.

Receptor purification and western blotting

Preparation of nuclear extracts from virus-infected cells followed by Ni^{2+}–NTA chromatography purification were performed essentially as described (10,11,13). Western blotting was performed as previously described (10). Signal detection was performed using the AP kit (Promega).

Oligonucleotides

The synthetic oligonucleotides used in this study comprised nuclear receptor binding sites (listed in Fig. 2A) and *Sac*I flanking sequences.

Electromobility shift assays

For the preparation of ^{32}P-labelled oligonucleotides, two complementary single-stranded oligos were annealed and incubated with $[γ-^{32}P]dATP$ and T4 polynucleotide kinase (BioLabs). Electromobility shift assays were performed as previously described (10). Off-rate experiments were performed by adding a 500-fold molar excess of specific oligonucleotides to the preformed protein–DNA complexes. Complexes formed during the band shift reactions were separated on pre-cooled, pre-run (1 h, 200 V) 4% polyacrylamide gels containing 0.25× TBE at 4°C and 200 V.

Transcription templates

The annealed RORβ-specific response elements were cloned into the *Sac*I site of plasmid TK38[380] (14), containing the *tk* minimal promoter –38 to +1 linked to a 380 bp G-less cassette. The template $4RE_1$ contains four copies of the annealed RE1
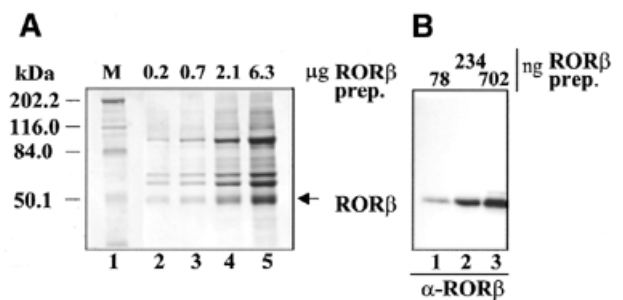

Figure 1. Analysis of the Ni^{2+}–NTA purified His_6–RORβ preparation. (**A**) Protein gel stained with Coomassie brilliant blue R-250. (**B**) Western blot analysis of the Ni^{2+}–NTA purified receptor preparation. M, molecular mass marker. The position at which His_6–RORβ migrates is indicated.

element cloned into the *Sac*I site of TK38[380]. The 5′-located RE1 element is in the antisense orientation, the three 3′-located elements in the sense orientation. In addition, the following templates were used: a RARβ[380] G-less construct (11), a TI[320] G-less construct (15) and the HIV-1 core promoter carrying five Gal4 recognition sites (16).

In vitro transcription assays

Aliquots of 10 ml packed cell volume (PCV) of HeLa cells and 3 ml PCV of Neuro2A cells were used to prepare crude nuclear extracts (17). The nuclear extracts had a final KCl concentration of 400 mM. A typical nuclear extract preparation had a protein concentration of 5–8 mg/ml. Transcription reactions were performed as described (10). The final KCl concentration in the transcription buffer for RXRα–His_6–RARα and Gal–VP16 transcription were set to 80 mM, for RORβ transcription to 50 mM. Aliquots of 4–8 μg crude nuclear extract preparations were used per reaction. A typical transcription reaction was performed by preincubating the samples for 40 min at 30°C in the absence of nucleotides. After addition of nucleotides the procedure was followed for up to 60 min reaction time. Single round transcription experiments were performed by adding Sarkosyl to a final concentration of 0.05% 30 s after nucleotide addition.

RESULTS

Expression of RORβ using the vaccinia virus system

To assess the transcriptional properties of RORβ, a histidine-tagged fusion protein, His_6–RORβ, was expressed in HeLa cells using the vaccinia virus expression system and purified over a Ni^{2+}–NTA column. The SDS–PAGE polypeptide pattern of a typical His_6–RORβ preparation revealed a polypeptide of ~50 kDa which was not present in preparations from HeLa cells infected with wild-type vaccinia virus (Fig. 1A and data not shown). Western blot analysis using a polyclonal antiserum raised against RORβ identified the 50 kDa polypeptide as RORβ (Fig. 1B). Apart from RORβ, other poly(His)-containing polypeptides present in vaccinia-infected as well as in non-infected HeLa cells bind to Ni^{2+}–NTA (Fig. 1A, lanes 2–5 and data not shown). The purity of His_6–RORβ was estimated to be 5–10%.
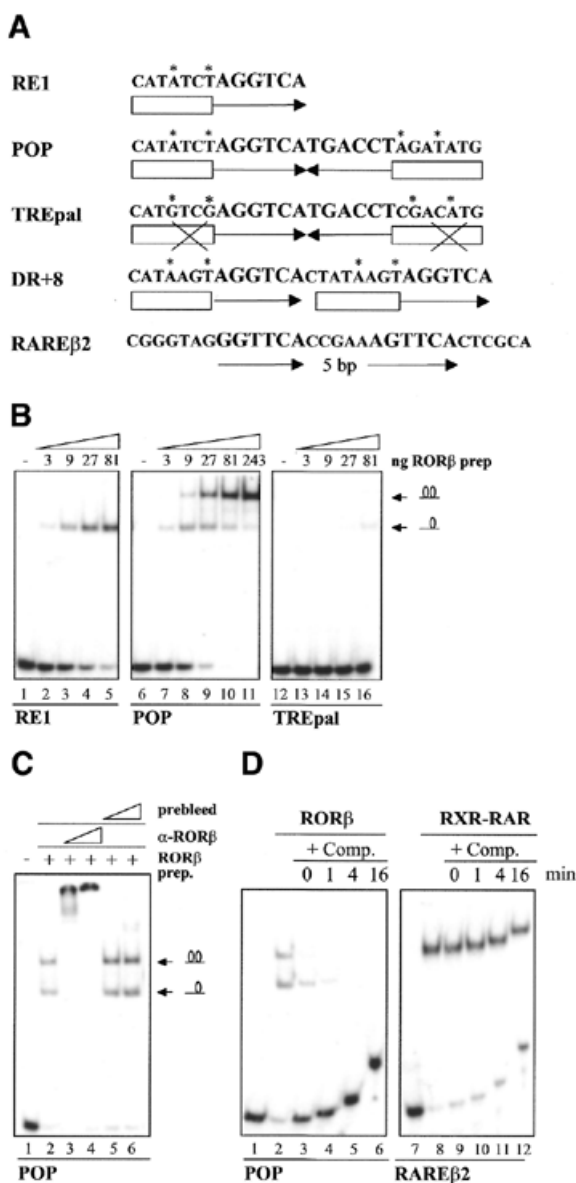
Figure 2. DNA binding behavior of RORβ. (**A**) Binding sites used in this study. The conserved nuclear receptor half-site is symbolised by an arrow, the 5′-extension of the half-site by a box. The nucleotides within the extensions that are critical for RORβ DNA binding are marked with an asterisk. (**B** and **C**) EMSA analysis of RORβ DNA binding to distinct binding sites, which are indicated below the experiments. (C) Aliquots of 27 ng RORβ preparation (lanes 2–6) and 0.1 or 1 μl of a RORβ-specific polyclonal antibody (lanes 3 and 4) or pre-bleed (lanes 5 and 6) serum were used. (**D**) Off-rate analysis of RORβ binding to a POP element and RXRα–RARα binding to a RAREβ2 element.

RORβ has a weak affinity for its DNA site

Since DNA binding of a transcription factor is a prerequisite for transcriptional activation, the DNA-binding properties of His$_6$–RORβ were analysed in electrophoretic mobility shift assays. Several oligonucleotides were used containing either a single or two consensus half-sites (AGGTCA) in a direct or palindromic configuration (Fig. 2A). Because DNA binding of RORα and RORβ reportedly depends on sequences 5′ of the consensus half-site (7,8,18), the ROR-responsive element

(RORE) used throughout the experiments contained a thymine at position –1 and an adenine at position –4 preceeding the nuclear receptor half-site. His$_6$–RORβ readily bound as a monomer to a single half-site (RE1) (Fig. 2B, left). Two complexes were obtained on POP, a binding site containing two half-sites in a palindromic configuration (Fig. 2B, middle). In agreement with published results (8,19), two distinct complexes were also detected using response elements consisting of two half-sites arranged as direct repeats with 7 or 8 spacing nucleotides (DR+7 and DR+8; data not shown). Both protein–DNA complexes were supershifted with a RORβ-specific antiserum, whereas the preimmune serum did not alter the mobility of the complexes (Fig. 2C). Binding of His$_6$–RORβ to TREpal, containing two palindromically arranged consensus binding sites without appropriate 5′-extensions, was very inefficient; a weak protein–DNA complex migrating at the position of a monomer was obtained only at high receptor concentrations (Fig. 2B, right).

Next, the relative affinity of His$_6$–RORβ for the RORE was assessed. For this purpose, off-rate experiments were performed and compared with those of the related RXRα–His$_6$–RARα heterodimer (Fig. 2D). In these assays vaccinia-expressed receptors were preincubated with labelled oligonucleotide probes to allow formation of protein–DNA complexes. Dissociation of the pre-formed protein–DNA complexes was measured as a function of time by loading aliquots onto a continuously running acrylamide gel at 0, 1, 4 and 16 min after addition of a 500-fold molar excess of specific cold competitor. While the RXRα–His$_6$–RARα heterodimer complex bound to the RAREβ2 element was not or only marginally affected by addition of excess competitor (Fig. 2D, right; 20), His$_6$–RORβ–DNA complexes formed on the POP element dissociated rapidly after addition of competitor (Fig. 2D, left). Similar results were obtained using other ROREs (RE1, DR+8 and DR+7) (data not shown).

Taken together, these data show that His$_6$–RORβ can bind to elements that contain either one or two extended binding sites. However, His$_6$–RORβ has a low affinity for the response elements tested compared with the affinity of the related nuclear receptor heterodimer RXRα–His$_6$–RARα for RAREβ2.

Transcriptional activation in Neuro2A and HeLa nuclear extracts

To assess the transcriptional activity of His$_6$–RORβ, we established an *in vitro* transcription system similar to that described for the RXRα–His$_6$–RARα heterodimer (10,11). The reporter construct contained a RORE in front of the TATA box of the *tk* promoter fused to a G-less cassette of 380 nt (Fig. 3A). A template with the adenovirus major late TATA box initiator fused to a G-less cassette of 320 nt (TI) served as an internal control, monitoring the basal transcriptional activity of the extracts. Since RORβ is expressed predominantly in neuronal tissues, nuclear extracts from the neuroblastoma cell line Neuro2A were used in initial experiments as a source of basal transcription factors and putative cofactors. For quantitative analysis, transcription levels were measured using a Phosphorimager.

His$_6$–RORβ preparations boosted the level of transcription up to 30-fold from the palindromic POP element-containing template in a concentration-dependent manner, whereas the
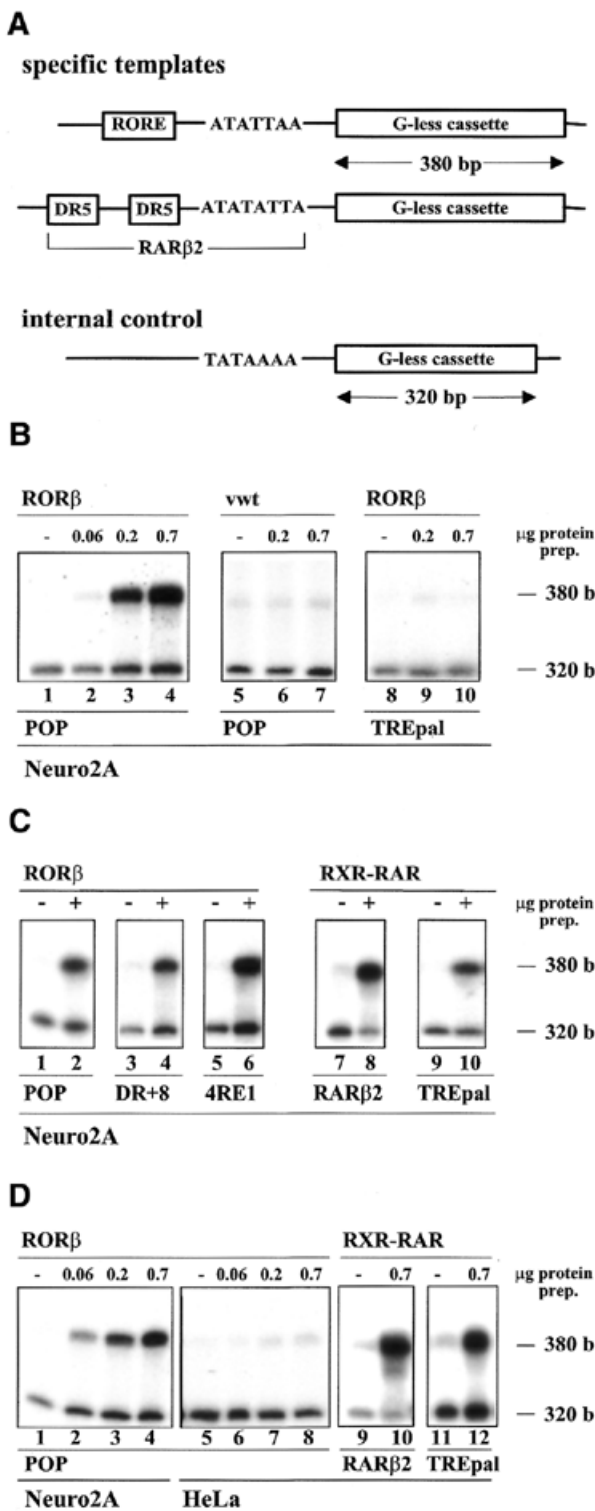
A

specific templates

B

C

D

Figure 3. RORβ is a strong transcriptional activator in Neuro2A nuclear extracts *in vitro*. (**A**) *In vitro* transcription templates used in this study. RORE, RORβ-specific response element. RARβ2, fragment of the RARβ2 promoter containing two DR5 elements. (**B–D**) *In vitro* transcription assays using Neuro2A (B–D) or HeLa (D) nuclear extracts. The vaccinia-expressed proteins used in the experiments are indicated above, the response elements of the *in vitro* transcription templates below the experiments. vwt, vaccinia wild-type nuclear extract. (C) Aliquots of 0.2 μg vaccinia-expressed His$_6$–RORβ and vaccinia-expressed RXRα–His$_6$–RARα were added to the *in vitro* transcription templates when indicated.

levels of transcription from the internal control were not or only marginally affected (Fig. 3B, lanes 1–4; Fig. 3C, lanes 1 and 2; Fig 3D, lanes 1–4). The increase in levels of transcription is specific for His$_6$–RORβ because control extracts derived from wild-type vaccinia virus-infected cells did not boost the level of transcription from the specific reporters (Fig. 3B, lanes 5–7). Furthermore, His$_6$–RORβ-dependent transcription was very low to undetectable when a reporter was used containing the TREpal element that did not support binding of RORβ (Fig. 2B right and Fig. 3B, lanes 8–10). Maximal transactivation by His$_6$–RORβ was observed with a template containing four extended binding sites (4RE1) (Fig. 3C and data not shown). Taken together, the ability of His$_6$–RORβ to bind to the ROREs correlates with its ability to mediate transcription through these elements.

Next, we wanted to compare the transcriptional activities of RORβ and RXRα–RARα. We previously showed that RXRα–His$_6$–RARα is a transcriptional activator in HeLa nuclear extracts when a transcription template was used that contained a fragment of the RARβ2 promoter with two natural DR5 elements (RAREβ2) (11). Therefore, *in vitro* transcription reactions were performed with His$_6$–RORβ and RXRα–His$_6$–RARα in Neuro2A and HeLa nuclear extracts.

RXRα–His$_6$–RARα strongly boosted transcription from the RARβ2 promoter-containing template in the neuronal extracts (Fig. 3C, lanes 7 and 8). In addition, RXRα–His$_6$–RARα activated transcription very efficiently from a template containing a TREpal binding site in front of the minimal *tk* promoter, which is identical to the POP–*tk* reporter except for the 4 bp flanking the palindromic element, the so-called 5′-extension (Fig. 3C, lanes 9 and 10). Loading the heterodimer with all-*trans* retinoic acid did not further enhance the level of transcription under the conditions used in these experiments (data not shown). The experiments revealed that RORβ and RXR–RAR are comparably efficient in transcriptional activation in Neuro2A nuclear extracts *in vitro*.

When the transcriptional activities of His$_6$–RORβ and RXRα–His$_6$–RARα were compared in HeLa nuclear extracts, only few transcripts instigated by His$_6$–RORβ could be detected using templates containing the POP binding site (Fig 3D, lanes 5–8). RORβ was also unable to activate transcription efficiently from templates containing two extended binding sites arranged as direct repeats (DR+7 and DR+8) or four extended binding sites (4RE1) (data not shown). In contrast, RXRα–His$_6$–RARα activated transcription very efficiently from RARβ2 (Fig. 3D, lanes 9 and 10), as well as from the TREpal template (Fig. 3D, lanes 11 and 12), indicating that the HeLa nuclear extracts were capable of supporting activated transcription.

Taken together, RORβ is a strong transcriptional activator in Neuro2A but not in HeLa nuclear extracts *in vitro*.

RORβ supports formation of functional preinitiation complexes

The insufficiency of RORβ to activate transcription in HeLa nuclear extracts prompted us to investigate whether RORβ is able to support the formation of an active preinitiation complex (PIC) in Neuro2A and HeLa nuclear extracts.

The formation of functional PICs can be measured by single round transcription assays (21). One commonly used method to restrict transcription to a single round *in vitro* is addition of

the anionic detergent Sarkosyl shortly after transcription reactions have been started by addition of nucleotides. Addition of a defined concentration of Sarkosyl prevents PIC assembly as well as formation of the first phosphodiester bonds, but not elongation of an initiated transcript (22). To define the optimal conditions in our experimental set-up (schematic presentation in Fig. 4A), the transcriptional activity of RORβ was analysed at different Sarkosyl concentrations. Addition of Sarkosyl to a final concentration of up to 0.025% during PIC assembly (a) or to a final concentration of up to 0.05% prior to formation of the first phosphodiester bonds (b) prevented transcription (Fig. 4B, lanes 1, 2, 7, 8, 13 and 14, and 3, 4, 9, 10, 15 and 16, respectively), indicating that these processes cannot take place under these conditions. Addition of 0.05% (final concentration) Sarkosyl 30 s after addition of nucleotides (c), i.e. after the formation of the first phosphodiester bonds, resulted in a low but appreciable level of transcription, consistent with only one round of transcription (Fig. 4B, lanes 17 and 18 and Fig. 4D). Addition of higher concentrations of Sarkosyl (0.08%) prevented transcription altogether (Fig. 4B, lanes 19–24). These results suggest that adding Sarkosyl to a final concentration of 0.05% immediately following addition of nucleotides limits transcription to a single round (single round condition). The underlying assumption of this interpretation is that formation of PICs is saturated after 40 min.

To test this hypothesis, *in vitro* transcription reactions with RORβ were preincubated under single round conditions for different time periods (Fig. 4C). In our experimental system 10–15 min preincubation was necessary to detect the first RORβ-dependent transcripts. The level of transcription did not further increase after 40 min, indicating that saturation of PIC formation was reached.

To assess the ability of RORβ to support single round transcription in HeLa and Neuro2A nuclear extracts, *in vitro* transcription experiments under single and multiple round conditions were performed with RORβ and RXRα–His$_6$–RARα. Experiments with the unrelated transcription factor Gal–VP16 served as additional controls. *In vitro* transcription experiments with His$_6$–RORβ were performed with templates containing either the 4RE1 or the POP binding site. His$_6$–RORβ behaved in a similar fashion on both templates. In the experiments presented templates with the 4RE1 binding site were used. The transcriptional activity of RXRα–His$_6$–RARα was analysed on templates containing a fragment of the RARβ2 promoter. In single round assays only transcription from the specific template was examined to avoid competition between the templates. To ensure reproducibility of the experiments, every experiment was repeated several times; the experiments presented are typical examples.

Figure 5 demonstrates that His$_6$–RORβ, RXRα–His$_6$–RARα and Gal–VP16 were able to support low but consistent single round transcription in Neuro2A (Fig. 5A) and HeLa (Fig. 5B) nuclear extracts (lanes 4, 8 and 12, respectively). Thus all three proteins, including RORβ, promoted preinitiation complex formation in Neuro2A and HeLa nuclear extracts.

Next, we determined the ratio of transcripts under multiple and single round conditions [indicated in Figs 4–6 as the ratio (mr)/(sr)] for His$_6$–RORβ, RXRα–His$_6$–RARα and Gal–VP16 in Neuro2A and HeLa nuclear extracts. In Neuro2A nuclear extracts, His$_6$–RORβ, RXRα–His$_6$–RARα and Gal–VP16 (Fig. 5A, lanes 1–4, 5–8 and 9–12, respectively) efficiently instigated
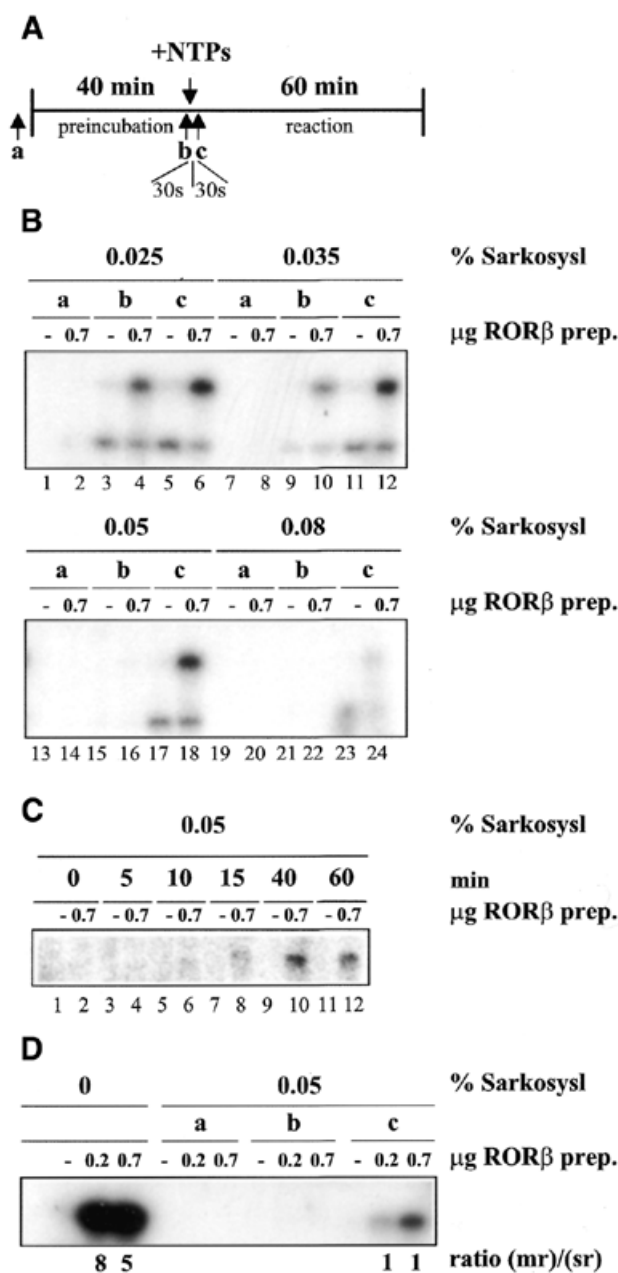

Figure 4. RORβ can efficiently support single round transcription *in vitro*. (**A**) Scheme of the *in vitro* transcription reaction. After a preincubation for 40 min, the transcription reactions were started by addition of nucleotide triphosphates (NTPs), followed by a further 60 min reaction. Sarkosyl was added at different time points either before preincubation (a), 30 s before (b) or after (c) addition of nucleotides. (**B**) *In vitro* transcription reactions in which Sarkosyl was added according the scheme of (A) to different final concentrations. (**C**) *In vitro* transcription reactions under single round conditions with different preincubation times. (**D**) *In vitro* transcription reactions under multiple round and single round conditions. The levels of transcription obtained when Sarkosyl was added 30 s after addition of nucleotides (single round conditions) were normalised to 1 for each RORβ concentration. The quotient between the amount of transcripts in a specific experiment and the amount of transcripts under single round conditions [ratio (mr)/(sr)] is indicated below the experiments.

transcription under multiple round conditions. In HeLa nuclear extracts, however, efficient transactivation under multiple round conditions could be observed for RXRα–His$_6$–RARα
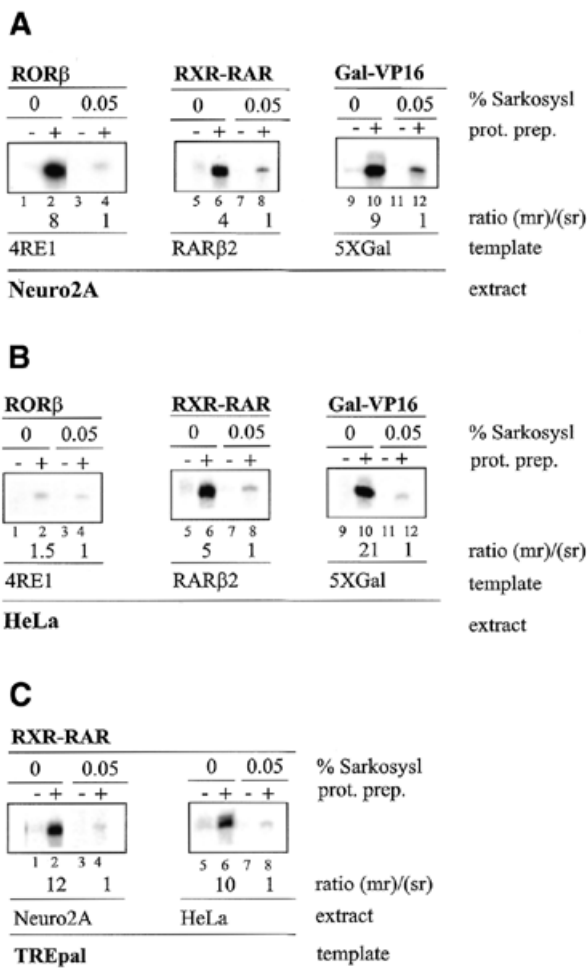
Figure 5. *In vitro* transcription reactions with RORβ, RXRα–RARα and Gal–VP16 under single and multiple round conditions using Neuro2A (**A**) and HeLa (**B**) nuclear extracts. (**C**) *In vitro* transcription reaction in Neuro2A and HeLa nuclear extracts with RXRα–RARα using an artificial template with a TREpal binding site. Aliquots of 0.2 μg RORβ or RXRα–RARα and 80 ng Gal−VP16 were added when noted. The levels of transcription obtained when Sarkosyl was added 30 s after addition of nucleotides (single round conditions) were normalised to 1 for each experimental set-up. The quotient between the amount of transcripts in a specific experiment and the amount of transcripts under single round conditions [ratio (mr)/(sr)] is indicated below the experiments.

Figure 6. RORβ-dependent transcription is insufficient in HeLa nuclear extracts. (**A**) *In vitro* transcription assays under single round conditions in Neuro2A and HeLa nuclear extracts with different preincubation times. (**B**) *In vitro* transcription reactions were preincubated for 40 min, started with nucleotides and then incubated under multiple round conditions for the indicated times. Transcription reactions in lanes 1, 2, 9 and 10 were performed under single round conditions with a reaction time of 60 min. (**C**) The same experimental set-up as in (**B**), but with longer reaction times. The ratios (mr)/(sr) are plotted against time. In all experiments 0.2 μg RORβ or RXRα–RARα were added when noted.

(Fig. 5B, lanes 5–8) and Gal–VP16 (Fig. 5B, lanes 9–12), but not for His₆–RORβ (Fig. 5B, lanes 1–4). The number of transcripts instigated by RORβ in HeLa nuclear extracts under multiple to single round conditions was approximately equal.

The observed difference in transcriptional behavior of His₆–RORβ and RXRα–His₆–RARα may have been caused by the differential requirements of the basal promoter used in these experiments, i.e. the minimal *tk* versus the RARβ2 promoter. To rule this out, the experiments were repeated for RXRα–His₆–RARα using an artificial template that contained a TREpal binding site in front of the minimal *tk* promoter. RXRα–His₆–RARα activated transcription efficiently from this template in Neuro2A as well as in HeLa nuclear extracts, indicating that the basal promoter composition of the templates did not influence the transcriptional behavior of the proteins
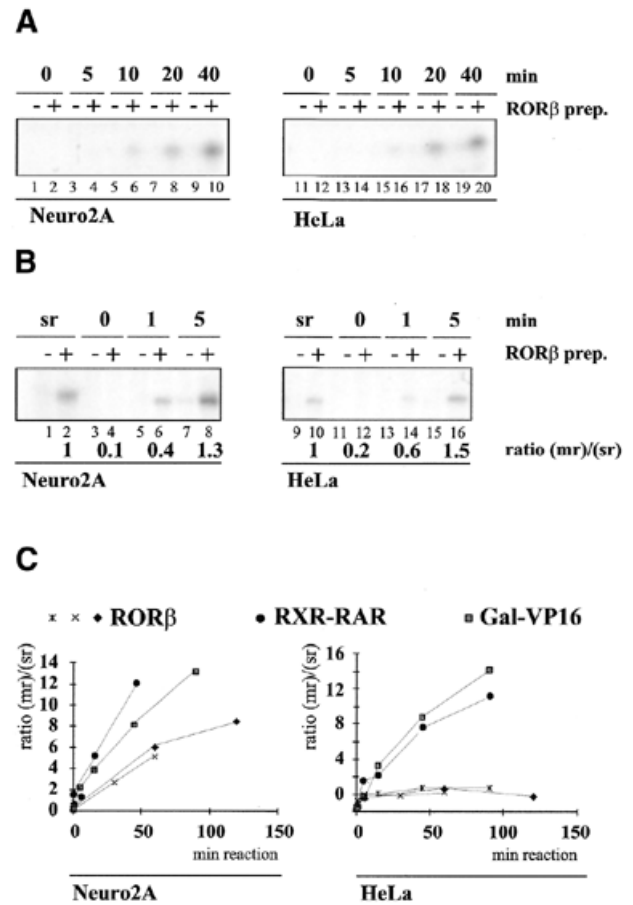
(Fig. 5C). Taken together, these experiments suggest a different transcriptional behavior of the two nuclear receptors RORβ and RXRα–RARα in HeLa nuclear extracts *in vitro*.

The observation that His₆–RORβ instigated significantly more transcripts in Neuro2A as compared with HeLa nuclear extracts prompted us to assess the rate at which His₆–RORβ boosts formation of functional preinitiation complexes in Neuro2A and HeLa nuclear extracts. As already demonstrated in Figure 4C, the rate of PIC assembly can be measured by performing experiments under single round conditions at different preincubation times. In Neuro2A as well as HeLa nuclear extracts, transcripts were synthesised in the presence of RORβ proportional to the time of preincubation (Fig. 6A). These experiments suggest that His₆–RORβ recruits PICs with equal efficiency in Neuro2A and HeLa nuclear extracts. Thus, the low amount of transcripts instigated by RORβ in HeLa nuclear extracts is not due to an extract-dependent insufficiency in the formation of functional PICs.

Next, we investigated the time required by His$_6$–RORβ, RXRα–His$_6$–RARα and Gal–VP16 to synthesise the equivalent of a single round of transcription to assess the rate at which transcripts are synthesised in HeLa and Neuro2A nuclear extracts. *In vitro* transcription experiments were performed under multiple round conditions and stopped at different times after addition of nucleotides (reaction times). RORβ-induced transcripts reached the equivalent of a single round transcription within 5 min after addition of nucleotides in Neuro2A (Fig. 6B, left) as well as in HeLa (Fig. 6B, right) nuclear extracts. This suggests that the rate of the synthesis of transcripts instigated by RORβ does not differ significantly in Neuro2A and HeLa nuclear extracts. Similar results were obtained for RXRα–His$_6$–RARα and Gal–VP16 (data not shown).

Similar experiments were performed with longer reaction times to assess the transcriptional efficiency of the proteins in the extracts. In Neuro2A nuclear extracts His$_6$–RORβ-instigated transcription resulted in doubling of the amount of transcripts every ~10–13 min, whereas no further increase in the number of transcripts could be observed in HeLa nuclear extracts (Fig. 6C). In contrast, RXRα–His$_6$–RARα and Gal–VP16 continuously produced transcripts in Neuro2A as well as in HeLa nuclear extracts.

Taken together, these experiments demonstrate that His$_6$–RORβ is equally efficient in supporting single round transcription in Neuro2A and HeLa nuclear extracts. However, *in vitro* the ability of RORβ to instigate transcripts is impaired in HeLa nuclear extracts.

DISCUSSION

RORβ is an orphan member of the nuclear receptor superfamily that belongs to subfamily 1, with T$_3$R and RAR as the most prominent members (1). Notwithstanding the extensive amino acid similarity between these receptors, our study suggests that the mechanism of transcriptional activation mediated by RORβ may differ from those of RAR and T$_3$R. Firstly, RORβ binds DNA as a monomer (8,10,19; this study), whereas T$_3$R and RAR require heterodimerisation with RXR (20,23). In agreement with these results, RAR is transcriptionaly active *in vivo* and *in vitro* only as an RXR heterodimer, while RORβ activates transcription as a monomer (10).

In off-rate experiments the heterodimers RXRα–RARα and RXRα–T$_3$Rα have high affinity for their cognate DNA-binding sites (20), whereas the affinity of RORβ (this study) and RORα (24) for a single, as well as a double, extended half-site is comparatively weak. Although the artificial binding sites used in this study have been shown to be optimal for RORβ DNA binding *in vitro* and for transcriptional activation *in vivo* (8,19), the existence of natural ROREs with high(er) affinities cannot be ruled out. Despite the different affinities for their cognate binding sites, RORβ and RXRα–RARα are equally efficient in the recruitment of functional PICs to DNA. It is important to note that PIC formation is a rather slow process. Several studies have determined the half-time of complex recruitment (i.e. the preincubation time required to obtain 50% of the final amount of transcripts generated under single round conditions) to be between 8 and 20 min (25–27). Saturation of PIC formation has been shown to require, as in our study, up to ~40 min (27).

Comparing the number of transcripts instigated by RORβ and RXRα–RARα under single versus multiple round transcription conditions (Fig. 5), we observed an insufficiency of HeLa nuclear extracts to support RORβ-dependent *in vitro* transcription. Whereas in Neuro2A nuclear extracts transactivation by RORβ resulted in continuous production of transcripts, in HeLa nuclear extracts the accumulation of transcripts ceased after 5 min. In contrast, RXRα–RARα and the unrelated transcription factor Gal–VP16 supported transcription in Neuro2A and HeLa nuclear extracts beyond the initial 5 min of incubation. This observation shows that HeLa nuclear extracts were in principle able to support efficient transactivation.

There are several explanations as to why transcription mediated by RORβ ceased after an initial short period of transcription in HeLa nuclear extracts. RORβ, in comparison with RXRα–RARα and Gal–VP16, might depend more strongly on a general factor(s) that is required for subsequent rounds of transcription and is limited in HeLa nuclear extracts. Putative candidates could be factors required for promoter clearance, such as the general transcription factor TFIIH (28,29), or for elongation of subsequent transcription rounds, such as the elongation factor SII (30).

Another intriguing possibility is that RORβ facilitates cofactor-dependent reinitiation in Neuro2A but not in HeLa nuclear extracts. Several *in vitro* studies have shown that reinitiation of transcription is fast as compared to the slow first round of transcription. In the first round of transcription, a preinitiation complex has to be recruited to the promoter, which appears to be a very slow process *in vitro*. At some promoters a committed complex consisting of TFIID and other factors such as TFIIA, TFIIB, TFIIH, TFIIE and Mediator has been detected after the initial polymerase escape (29,31–34). Such a committed complex might act as a scaffold for rapid formation of subsequent reinitiation complexes. Whether the continued presence of an activator is required to stabilise such a complex is unclear (27,31,35) and may depend on the transcription factor and template (29). Several activators, such as the heat shock factor or estrogen receptor, have been shown to enhance not only PIC assembly but also the rate of reinitiation in cell-free systems (31,36). RORβ might instigate transcription efficiently in Neuro2A but not in HeLa nuclear extracts due to the presence of an extract-specific factor(s) in Neuro2A nuclear extracts that facilitates RORβ-dependent reinitiation of transcription. Such a factor might be a cell type-specific homologue of TBP, such as TRF (37), or a cell type-specific cofactor involved in reinitiation. Alternatively, the impaired ability of RORβ to reinitiate transcription in HeLa nuclear extracts could be caused by the presence of an inhibitory factor in HeLa nuclear extracts that specifically blocks RORβ-dependent reinitiation.

The analysis of reinitiation mechanisms is difficult since it requires proof that initiation of the second and later rounds derive from the promoter already used in the first round of transcription, i.e. it has to be excluded that the transcripts synthesised at a later stage of incubation originate from previously untranscribed promoters. This is of particular importance since several studies have demonstrated that in cell-free transcription assays only a few per cent of the promoters are transcribed (21). The experiments shown in this study do not address the reinitiation mechanism and further experiments are necessary to analyse whether the transcripts produced in the

first and subsequent rounds of transcription are derived from the same promoters.

The presence of a cell type-specific transcription mechanism seems likely in the light of other studies. Transient transfection studies have shown that the ligand-binding domains of RORα (24) and RORβ (19) display a neuronal-specific transcriptional activity when they are fused to a Gal4 DNA-binding domain. In addition, a neuronal-specific cofactor for RORβ has recently been identified (38). This cofactor, called NIX1, does not activate but inhibits RORβ-dependent transactivation when overexpressed in transient transfection assays.

In summary, we have demonstrated that the orphan nuclear receptor RORβ is a comparatively strong transcriptional activator *in vitro* when considering its low affinity for ROREs. RORβ is as equally efficient as such strong DNA-binding factors as RXRα–RARα and Gal–VP16 in PIC stabilisation and continuous transcriptional activation in Neuro2A nuclear extracts. We further showed that RORβ, in contrast to RXRα–RARα and Gal–VP16, cannot instigate continuous transcription in HeLa nuclear extracts. This observation suggests a different (co)factor requirement for transactivation by members of the NR1 subfamily of nuclear receptors. Fractionation and further characterisation of nuclear extracts needs to be pursued to gain insight into the mechanism of transactivation by the orphan receptor RORβ.

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