MOLECULAR AND CELLULAR BIOLOGY, June 1991, p. 3379–3383
0270-7306/91/063379-05$02.00/0

Vol. 11, No. 6

The Glucocorticoid Receptor Binds to a Sequence Overlapping the TATA Box of the Human Osteocalcin Promoter: a Potential Mechanism for Negative Regulation

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Received 14 December 1990/Accepted 18 March 1991

Expression of the human osteocalcin promoter is negatively regulated by glucocorticoids in vivo. In vitro DNase I and exonuclease III footprinting analysis showed binding of purified glucocorticoid receptor in close proximity to and overlapping with the TATA box of the osteocalcin gene. These results imply competition or interference with binding of the TATA box-binding transcription factor IID as a mechanism of repression of this gene by glucocorticoids. In support of this notion, point mutation analysis of the receptor binding site indicated that flanking nucleotides and not the TATA box motif per se were important for receptor interaction. Moreover, DNA binding competition assays showed specific binding of the receptor only to the TATA box region of the osteocalcin gene and not to the corresponding region of an immunoglobulin heavy-chain promoter.

The in vivo effects of steroid and steroidlike hormones are mediated by specific receptors belonging to a large superfamily of ligand-dependent transcription factors and are important for homeostasis, growth, and development (8, 11, 29). Functional analysis has shown that these receptors modulate the transcriptional activity of target promoters by interacting with inducible regulatory sequences (responsive elements). Moreover, the glucocorticoid receptor stimulates transcription from some promoters (2, 29) while down-regulating others (1, 3, 7, 9, 10, 24, 27).

The gene for the noncollagenous bone protein osteocalcin (also known as gla protein) is expressed in osteoblasts (reviewed in reference 12; the human osteocalcin gene is described in reference 4) and is transcriptionally regulated by a number of hormones and vitamins, as has been shown by transient and stable transfection experiments in the rat sarcoma cell line ROS 17/2.8, which has an osteoblast phenotype (15, 18, 25). It has been shown that the active metabolite of vitamin D [1,25$(OH)_2D_3$] induces osteocalcin gene transcription while glucocorticoids repress both basal-level transcription and vitamin D-induced levels (18). The DNA sequences mediating the vitamin D response have been located to nucleotides −513 to −493 (15, 18), whereas the negative effect of glucocorticoids has been shown to be mediated through the promoter region between nucleotides −196 and +34 relative to the transcription start site (18).

Here we demonstrate that the glucocorticoid receptor recognizes in vitro a sequence motif overlapping with the TATA box, indicating that the receptor may repress the activity of this promoter by interference with binding of the general transcription factor TFIID and associated proteins.

Identification of a glucocorticoid receptor-binding site in the human osteocalcin promoter. As reviewed above, sequences mediating glucocorticoid repression of the osteocalcin promoter are located within 200 nucleotides immediately upstream of the transcription start site (18). To identify target sequences for the glucocorticoid receptor within the osteocalcin promoter, we performed DNase I and exonuclease III footprinting experiments using purified receptor protein (Fig. 1). Plasmid pOSCAT1 (18) was cleaved with restriction enzymes as schematically indicated in Fig. 2 to generate fragments spanning sequences from −344 to +30 and −64 to +30, respectively, of the osteocalcin promoter. These fragments were asymmetrically ^{32}P labeled, incubated with various amounts of purified rat liver glucocorticoid receptor (28), and then subjected to either DNase I or exonuclease III footprinting experiments as described previously (21, 26).

DNase I footprinting analysis demonstrated that a region extending from −35 to −14 in the upper and lower DNA strands was protected from DNase I digestion following addition of glucocorticoid receptor (Fig. 1A and B). At higher protein concentrations, this binding site was extended to encompass nucleotides −11 to −3 primarily on the lower strand (Fig. 1A, lane 3; B, lanes 4 and 5). However, since this footprint varied in intensity from experiment to experiment (compare Fig. 1A, 1B, and 3B), it appears to represent nonspecific binding to a G+C-rich sequence motif. No further sequences between −344 and +30 were protected from DNase I digestion even at glucocorticoid receptor concentrations exceeding 2 pmol (data not shown).

The 3′ boundary of the glucocorticoid receptor-binding site on both DNA strands was determined by exonuclease III footprinting. The assay was performed as described previously (26) in the presence of 5 mM $MgCl_2$. The glucocorticoid receptor induced multiple terminations of exonuclease III digestion on the lower strand of a promoter fragment spanning nucleotides −64 to +30: one major termination site was detected at position −38, whereas two minor ones were detected at positions −36 and −34, respectively (Fig. 1C, lanes 6 and 7). On the upper strand of the promoter, the receptor induced an exonuclease III termination site at approximately −12 (Fig. 1C, lane 2). At a higher protein concentration (1 pmol), a second termination point at +5 was induced (lane 3). The diagram in Fig. 2 summarizes the DNase I and exonuclease III footprinting experiments and shows that the receptor-dependent exonuclease III termination sites were all detected at the boundaries of the

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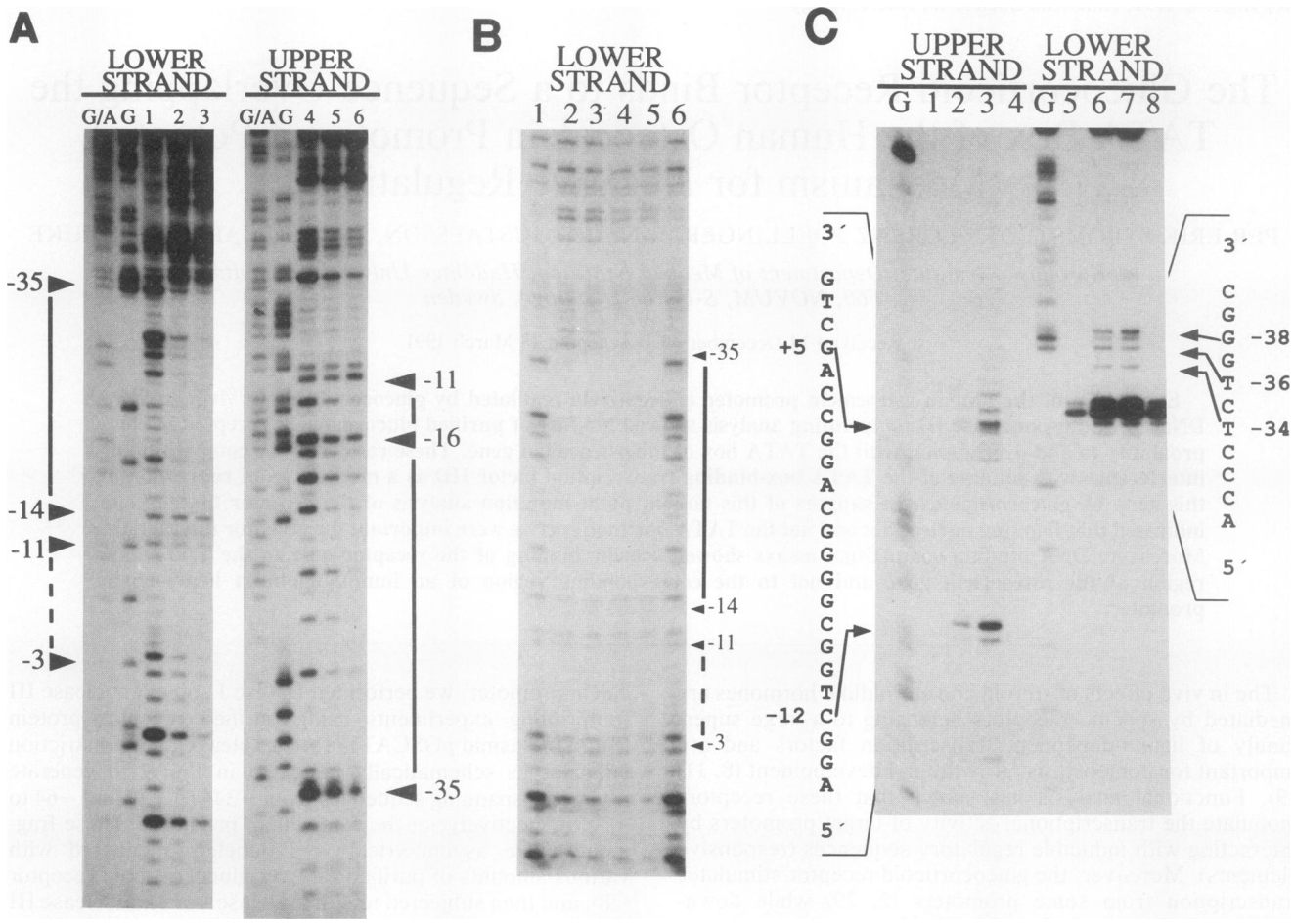

FIG. 1. DNase I footprinting and exonuclease III footprinting analysis of glucocorticoid receptor-binding sites in the osteocalcin promoter. DNA binding reactions containing purified glucocorticoid receptor were carried out in parallel on both the upper and lower strands of the human osteocalcin promoter. Positions of glucocorticoid receptor-dependent DNase I footprints are indicated at the sides of the autoradiographs by vertical bars relative to the point of transcription initiation. (A) The probe used was end labeled with ^{32}P at the *Sph*I site (−64) or the *Xho*I site (in chloramphenicol acetyltransferase reporter DNA), incubated in the absence (lanes 1 and 4) or presence of 0.5 pmol (lanes 2 and 5) or 1.0 pmol (lanes 3 and 6) of glucocorticoid receptor, digested with DNase I. Maxam-Gilbert (17) sequencing reactions (G/A and G) of the labeled fragment are shown in parallel lanes. (B) The fragment spanning from −344 to +30 was end labeled at the *Xho*I site, incubated in the absence (lanes 1 and 6) or presence of 0.25, 0.50, 1.0, or 2.0 pmol of glucocorticoid receptor (lanes 2 to 5, respectively), and subjected to DNase I digestion (see above). (C) Exonuclease III footprinting. The osteocalcin promoter was labeled at either the *Xho*I or *Sph*I site, incubated in the absence of (lanes 1, 4, 5, and 8) or presence of 0.5 pmol (lanes 2 and 6) or 1.0 pmol (lanes 3 and 7) of glucocorticoid receptor, and subsequently digested with exonuclease III. The arrows and the numbers indicate glucocorticoid receptor-induced termination sites of exonuclease III digestion.

DNase I footprints. No further glucocorticoid receptor-dependent termination point of exonuclease III digestion was found upon analysis of the larger DNA fragment spanning from −344 to +30 (data not shown).

Computer searches for consensus glucocorticoid-responsive elements (GREs) revealed a sequence similar to a positive GRE overlapping with the TATA box. This sequence motif shares 9 nucleotides out of 12 (Fig. 2) with a positive consensus GRE suggested by Jantzen et al. (14). No other sequence with a similar or higher degree of homology to the consensus GRE could be found within the 0.37-kb fragment of the 5′-flanking sequence of the human osteocalcin promoter. More importantly, the sequence motif identified by computer analysis is centered within the receptor-dependent footprint.

Specificity of glucocorticoid receptor binding to the osteocalcin promoter. To examine the specificity of the glucocorticoid receptor binding to the basal promoter element of the osteocalcin gene, we performed DNase I and gel mobility shift competition experiments. In the DNase I experiments, we used fixed amounts of purified glucocorticoid receptor (1.0 pmol) and the ^{32}P-labeled fragment extending from position −344 to +30. We used 20-fold (Fig. 3B, lanes 4, 6, 8, and 10) and 50-fold (lanes 5, 7, 9, and 11) molar excesses of unlabeled oligonucleotide to compete for glucocorticoid receptor binding. As diagrammed in Fig. 3A, the oligonucleotides contained wild-type or mutant sequence motifs derived from the osteocalcin promoter sequence protected in the DNase I and exonuclease III footprinting experiments or sequences unrelated to the human osteocalcin promoter sequence. The oligonucleotide containing a synthetic GRE derived from the rat tyrosine aminotransferase gene (tatGRE; Fig. 3B, lanes 4 and 5) and an oligonucleotide (oGREt; Fig. 3B, lanes 8 and 9) carrying clustered point mutations of

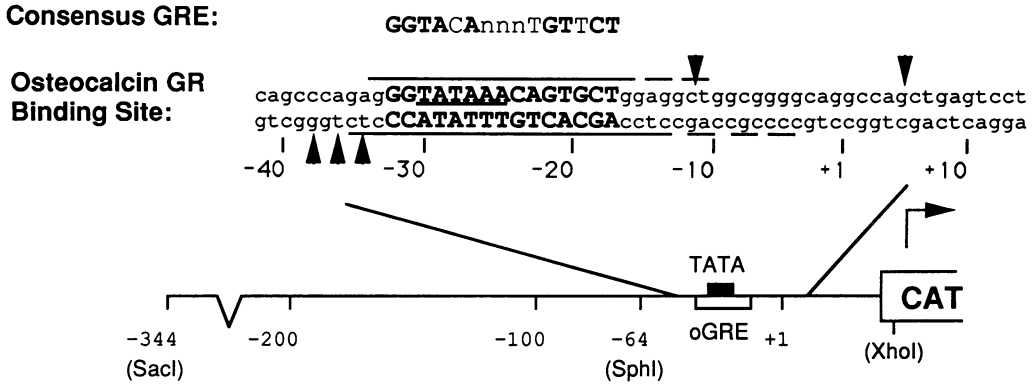

FIG. 2. Diagram of the human osteocalcin promoter and summary of the footprinting data. The diagram shows the 0.37-kb DNA fragment examined, containing the human osteocalcin promoter (from −344 to +30) and the chloramphenicol acetyltransferase (CAT) reporter DNA (18). The restriction sites used for end labeling of relevent fragments are indicated. Sequences of the TATA box and GRE motif (TATA box underlined) are highlighted in bold letters. Lines above and below the DNA sequences indicate nucleotides that were protected on each strand from DNase I digestion in the presence of receptor. Arrows above and below the sequences indicate the 3′ borders of the receptor-binding site as determined by exonuclease III footprinting experiments. For comparison, a consensus GRE (14) sequence is shown above. Nucleotides which share positional homology with the osteocalcin GRE (oGRE) are shown in bold letters.

the TATA box motif in the osteocalcin receptor-binding site both competed for binding of the glucocorticoid receptor. In contrast, an oligonucleotide (oGREg) carrying mutations within the GRE motif but an intact TATA box sequence did not compete for binding (Fig. 3B, lanes 10 and 11). Similarly, an oligonucleotide (igTATA; H^+O^+ in reference 22) spanning the octamer and TATA box elements of an immunoglobulin heavy-chain promoter (Fig. 3B, lanes 6 and 7) did not compete for binding. These experiments demonstrate that the degenerated GRE motif and not the TATA box motif itself is critical for receptor interaction in the basal promoter of the osteocalcin gene.

These results were confirmed by gel mobility shift experiments. To assess the relative affinity of the glucocorticoid receptor for the oGRE sequence, gel mobility shift experiments were carried out essentially as described previously (13). A ^{32}P-labeled tatGRE oligonucleotide was incubated with purified glucocorticoid receptor in the presence or absence of increasing concentrations of unlabeled competitor DNA. Oligonucleotides oGRE, containing the wild-type glucocorticoid receptor-binding site of the osteocalcin promoter, tatGRE, and igTATA were used as competitors. The DNA binding reactions were performed at ambient temperature for 15 min, and bound and free DNAs were electrophoretically separated on a 4.5% (wt/vol) native polyacrylamide gel. Figure 4A shows the receptor-dependent GRE complex (lane 2, complex indicated by an arrow). Gel mobility shift competition experiments confirmed that GRE complex formation was due to specific receptor-DNA interaction. Thus, receptor-GRE interaction was inhibited by the addition of an excess of unlabeled probe (Fig. 4A; compare lanes 2, 3, 4, and 5) or oligonucleotide oGRE (compare lanes 2, 9, 10, and 11), whereas addition of identical molar excesses of the igTATA oligonucleotide had no effect on receptor-GRE complex formation (compare lanes 2, 6, 7, and 8). Quantitation of the DNA binding competition experiments indicated that the tatGRE sequence exhibited a two- to fourfold-higher relative affinity for the receptor than did the oGRE fragment (Fig. 4B). However, in vivo both the dexamethasone-induced down-regulation of osteocalcin and the induction of tyrosine aminotransferase gene expression are detected at very similar concentrations of hormone (14, 18). Finally, the gel mobility shift competition experiments demonstrated that a TATA element from an unrelated gene did not compete for binding of the receptor.

A glucocorticoid receptor-binding site overlaps with the TATA box of the osteocalcin promoter. Using DNA footprinting techniques and gel mobility shift assays, we have identified within the osteocalcin promoter a sequence motif which is specifically recognized in vitro by the glucocorticoid receptor. This element spans nucleotides −35 to −14 relative to start site, overlaps with the TATA box, and contains a degenerate GRE consensus motif. Moreover, point mutation analysis demonstrates that the receptor-binding motif is distinct from the TATA box motif. Taken together, these results suggest that glucocorticoid-induced repression of osteocalcin gene expression may be mediated by binding of the glucocorticoid receptor to basal regulatory elements of the osteocalcin promoter, thereby resulting in interference or competition with binding of basal transcription factors such as TFIID and associated proteins.

Transcription interference as a model for repression of gene expression. Repression of transcriptional activity by competition between different transcription factors for binding to overlapping sequence motifs has been shown to be an important mechanism of transcriptional repression in eukaryotes (reviewed in reference 16 and 23). For instance, the glucocorticoid receptor has been shown to bind to a sequence overlapping with a cyclic AMP-responsive element in a promoter which is negatively regulated by glucocorticoids (1). Relevant to the present results, repression of transcriptional activity by interference with the basal transcription factors has recently been demonstrated for the engrailed factor, a homeobox protein important for temporal and spatial regulation of gene expression during *Drosophila* development (19, 20). In these studies, engrailed has been shown to compete with TFIID for in vitro binding to the TATA box of the *Drosophila* hsp70 promoter. More importantly, this competition at the TATA box results in repression of the promoter, as assessed by in vitro transcription analysis. In line with these observations, it has been shown that different forms of thyroid hormone receptor mediate negative regulation of the thyroid-stimulating hormone α subunit and growth hormone genes, respectively, via an

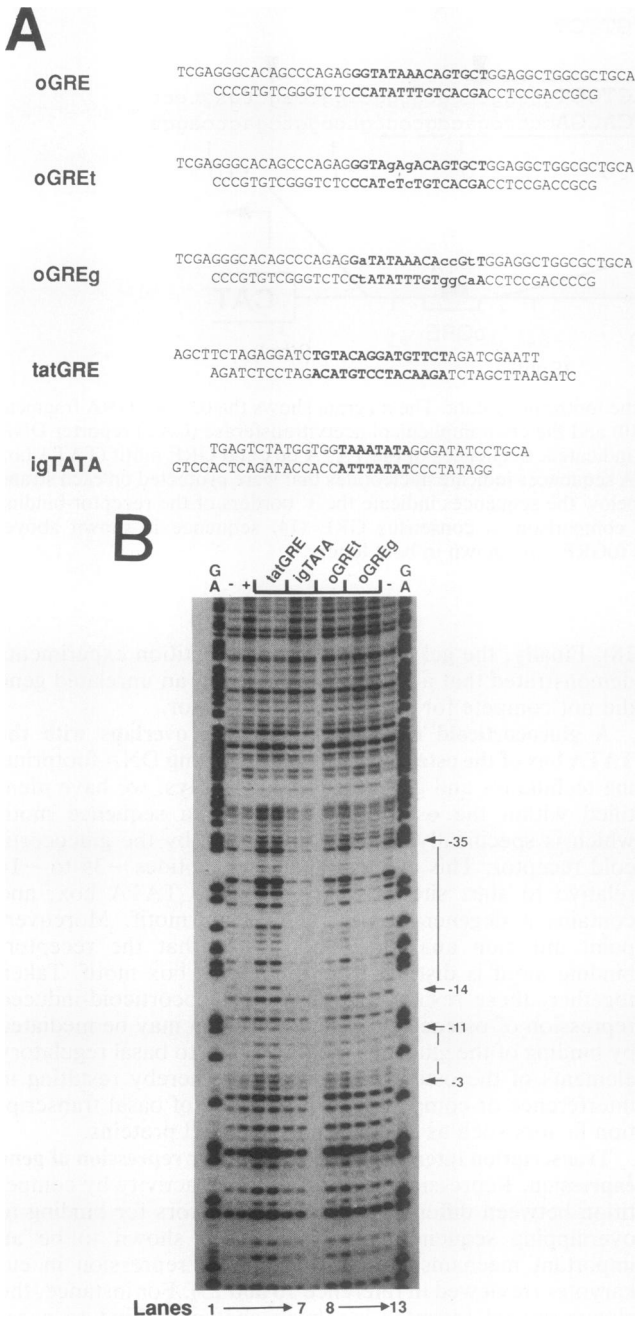

FIG. 3. Point mutation analysis of the glucocorticoid receptor-binding site in the osteocalcin reporter. (A) Sequences of synthetic fragments used as competitor DNAs in glucocorticoid receptor-binding reactions: oGRE, the wild-type sequence of the human osteocalcin promoter protected in DNase I experiments; oGREt, the oGRE sequence carrying point mutations within the TATA box motif; oGREg, the oGRE sequence carrying point mutations within the GRE motif; tatGRE, sequence of the GRE-2 of the rat tyrosine aminotransferase gene (14); igTATA, sequence of the TATA box from the BC11 immunoglobulin heavy-chain promoter (22). Lowercase letters indicate mutated nucleotides within the oGRE sequence. (B) DNase I footprinting competition experiments. DNA binding reactions were assembled with the ^{32}P-labeled *Sac*I-*Sph*I fragment of the human osteocalcin promoter (see Fig. 2) and purified glucocorticoid receptor (1.0 pmol) in the absence or presence of a 20- or 50-fold molar excess of the indicated unlabeled oligonucleotides.

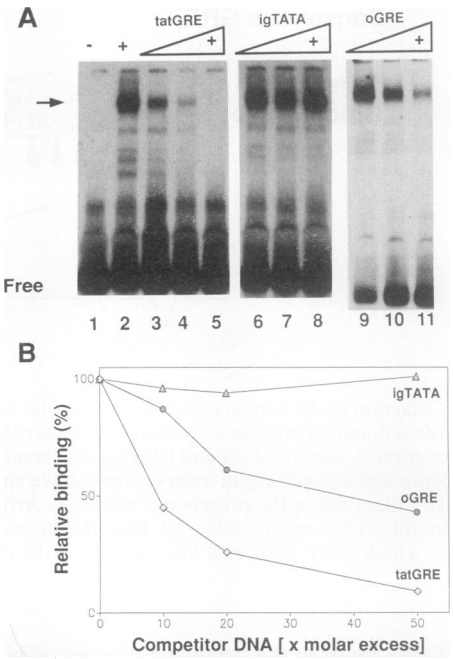

FIG. 4. Relative affinity of the glucocorticoid receptor for the oGRE sequence motif. (A) ^{32}P-labeled tatGRE was incubated with 0.2 pmol of purified glucocorticoid receptor in the absence (lane 2) or presence of increasing concentrations of the competitor oligonucleotides indicated (lanes 3 through 11). The major glucocorticoid receptor-induced complex is indicated by an arrow. Competitor DNA was added in a 10-fold (lanes 3, 6, and 9), 20-fold (lanes 4, 7, and 10), or 50-fold (lanes 5, 8, and 11) molar excess relative to the radiolabeled probe. (B) Bands representing the glucocorticoid receptor-induced complex were excised from vacuum-dried gels and quantitated by liquid scintillation counting.

element immediately downstream of the TATA box, indicating that this negative regulation could involve displacement of the TFIID (5, 6). However, in both of these promoters, it is not yet known whether the location of the receptor-binding site is close enough to the TATA box to perturb binding of TFIID. In the present study we demonstrate that a binding site for the glucocorticoid receptor in a negatively regulated promoter actually overlaps with that of TFIID, strongly arguing that binding of the glucocorticoid receptor and the TFIID may be mutually exclusive. Examination of this possibility in a functional assay and elucidation of the mechanism underlying negative regulation of the osteocalcin promoter by the glucocorticoid receptor will require the development of a reconstituted transcription system which uses both purified basic transcription factors and purified receptor.

We are very grateful to Agneta Andersson for technical expert assistance. We thank N. A. Morrison (Garvan Institute for Medical Research, Sydney, Australia) for providing plasmid pOSCAT1 and for discussion throughout the work.

This study was supported by grants from the Swedish Society for Medical Research, Bengt Lundqvists Minne, the Swedish Cancer Society (2171-01XAB), and the Swedish Medical Research Council (2819, 8557). P.-E.S. and J.C.-D. are recipients of research fellowships from the Swedish Medical Research Council.

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