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THE 5' FLANKING REGION OF THE HUMAN pS2 GENE MEDIATES ITS TRANSCRIPTIONAL ACTIVATION BY ESTROGEN IN MCF-7 CELLS

Margo ROBERTS[‡*], John WALLACE[•], Jean-Marc JELTSCH and Meera BERRY

Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Unité 184 de Biologie Moléculaire et de Génie Génétique de l'INSERM, Institut de Chimie Biologique - Faculté de Médecine 67085 STRASBOURG Cédex - France

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The pS2 gene is transcriptionally induced by oestrogen in the human breast cancer cell line MCF-7. We demonstrate here that the 5' flanking sequences (-3000 to +10 bp) of the pS2 gene possess the properties of an oestrogen-inducible promoter. Interestingly, this oestrogen induction could not be demonstrated in transient transfection assays in MCF-7 cells, but only in stably transformed MCF-7 cells, which suggests that some factors responsible for oestrogen induction may be present in limiting amounts in these cells and absent in HeLa cells.

The human breast carcinoma cell line MCF-7 (1) contains both oestrogen and progesterone receptors (2,3) and exposure of these cells to oestrogen results in the induction of certain mRNAs, proteins and secretory growth factors (4,5). We have previously reported the isolation of a cDNA clone, termed pS2, which corresponds to a 600 nucleotide (nt) mRNA species induced by oestradiol treatment of steroid hormone-deprived MCF-7 cells (6,7). Oestradiol (but not other steroid hormones such as progestins, glucocorticoids, or androgens) induces pS2-mRNA rapidly and directly at the transcriptional level via a mechanism which is independent of de novo protein synthesis (8). Recently we described the isolation and characterization of the human pS2 gene together with its flanking sequences from both the MCF-7 cell line and human placenta (11). In order to delineate those sequences of the human pS2 gene which mediate its transcriptional response to oestrogen, we have employed two approaches. The oestrogen-dependent expression of modified derivatives of the pS2 gene was investigated firstly in transient

Present addresses :
‡ Yale University - Dept. Biology, Kline Biology Tower, PO Box 6666 - New Haven, Conn. - USA.
• Purdue University - Dept. Botany and Plant Pathology, West Lafayette, Ind. 47907 - USA.
* To whom all correspondence should be sent.

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assays involving transfection into MCF-7 and HeLa cells, where pS2 mRNA is not detectable, and secondly in clonal populations of MCF-7 cells in which the recombinants were stably integrated into the cell genome.

MATERIALS AND METHODS

Plasmid constructions (see Fig. 1 and 2).

pS2M, the pBR322 based recombinant containing the entire pS2 gene of MCF-7 origin (9), was the parental plasmid. The SacI site present in the 3' untranslated region of exon 3 was changed to an unique BglII site after SacI digestion, S_1 nuclease treatment, Klenow repair and addition of a BglII linker. A 60 bp Sau 3AI fragment isolated from the SV40 early region (SV40 coordinates 4713-4773) was then inserted in the created BglII site to generate pS2MI. pS2MIE was derived from pS2MI by insertion of a BamHI-PvuII fragment encompassing the SV40 enhancer (coordinates 101-270, see ref. 13), into the unique SalI site, present in the pBR322 sequence, after Klenow repair.

The BamHI-HinfI fragment of the pS2 promoter region (coordinates -90 to +10) was inserted, after Klenow repair, in the SmaI site of pHP34 (18) to yield pHP34-90. The pS2 3 kb SmaI fragment (coordinates ∿ -3000 to -84) was inserted in the SmaI site of pHP34-90 (which is the Sma site at position-84 in the pS2 gene) in the correct orientation to restaure the 3 kb pS2 promoter region, generating pHP34-3000. The replacement of SV40 T-antigen sequences (SV40 coordinates 5171 to 2666) in the plasmid PEMP (19) by the 1 kb Tn5-neo gene sequence (from BglII to SmaI, see ref. 12) yielded pMRNeo.pMRNeo-3000 was obtained by cloning the 3 kb pS2 promoter region, excised from pHP34-3000 with EcoRI, into the BamHI site of pMRNeo after Klenow repair. This promoter region can direct the transcription of the "neo" gene. pMRNeo and pMRNeo-3000 contain a SV40 leader sequence (SV40 coordinates 5226-5171) and SV40 polyadenylation signal and polyA addition sites (SV40 coordinates 2666-2533).

Analysis of transfectants

Plasmids were introduced into steroid-deprived MCF-7 cells by calcium phosphate-mediated transfection (10). MCF-7 cells were grown in medium containing steroid hormone-stripped foetal calf serum (withdrawn medium) for at least 5 days prior to transfection. Eight to 16 hours after transfection, half of the cells were exposed to 10^{-8}M oestradiol, and cytoplasmic RNA was isolated 24 hours later. A similar protocol was followed for HeLa cells grown in normal medium.

Extraction of cytoplasmic RNA, agarose gel electrophoresis and Northern hybridization were carried out as previously described (8, 11). Probes used in the analysis of stable MCF-7 transfectants were a 1 kb "neo" BglII-SmaI fragment isolated from pAG60 (12), a 150 bp PstI fragment isolated from plasmid 3A5 (4), and a 485 bp BamHI-ClaI pS2 cDNA fragment isolated from pSVES1 (6).

The transient expression of transfected plasmids were assayed by S1 nuclease quantitation of cytoplasmic RNA. Single-stranded probes were derived from the M13 recombinants, M13mp10pS2 and M13mp18pS2MI. M13mp10pS2 was constructed by insertion of a 385 bp Sau3A fragment isolated from the 11.8 kb BglII fragment of the genomic clone pS2M (9), and encompassing 202 bp of exon 3 plus 183 bp of the 5'-flanking intron of the native pS2 gene, into the BamHI site of M13mp10. Similarly, M13mp18pS2MI was contructed by insertion of a 432 bp HaeIII fragment isolated from pS2MI (Fig. 1A), and spanning 216 bp of modified exon 3 (i.e. including the 60 bp I insert) plus 216 bp of the 5'-flanking intron, into the SmaI site of M13mp18. Synthetic oligonucleotides (16-mer) complementary to unique 16 nt sequences present in the RNA coding strand of either the unmodified exonic region of M13mp10pS2 or of the 60 bp insert I of M13mp18pS2 MI were hybridized to the appropriate single-stranded M13 template. After primer extension and digestion with EcoRI (which cuts in the polylinker of the M13 vector, 5' to the cloned fragment in both cases), the single stranded DNA probes were purified by gel electrophoresis as

previously described (13), and 5'-end-labelled with [γ-^{32}P]-ATP using T4 polynucleotide kinase.

RESULTS AND DISCUSSION

The transient expression of the transfected pS2MI and pS2MIE plasmids and of the endogenous pS2 gene were assayed by S1 nuclease quantitation of cytoplasmic RNA using single-stranded probes homologous to the final intron/-exon boundaries of both the native pS2 and modified pS2MI genes (Fig. 1A and B). The probe employed for analysis of pS2MI and pS2MIE (pS2MI with the SV40

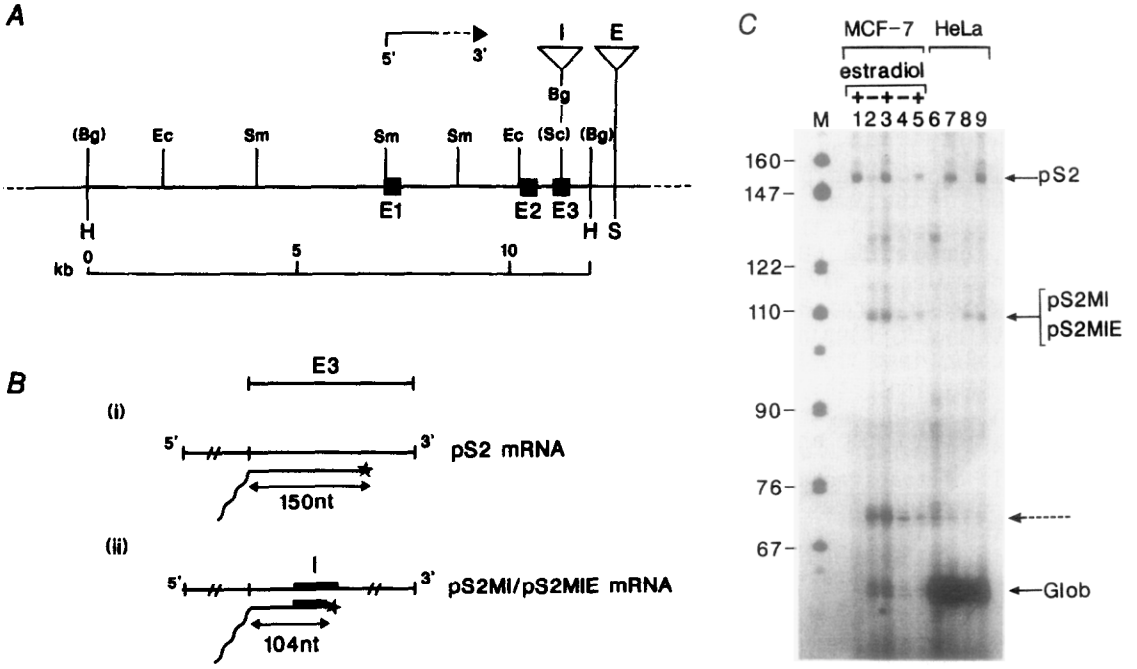

Figure 1. (A) Maps of pS2MI and pS2MIE. I : 60 bp insert from SV40, E : SV40 enhancer (not drawn to scale). E1, E2, E3 :exons of the pS2 gene. BglII=Bg, EcoRI=Ec, HindIII=H, SalI=S,SacI=Sc, SmaI=Sm. Sites in parenthesis were destroyed during plasmid constructions.

(B) Probes for discrimination of pS2 (i) and pS2MI/pS2MIE (ii) encoded transcripts in S_1 nuclease analysis (not drawn to scale). Single stranded DNA probes are composed of an intron part (weavy lane) and an exon E3 part (—). Sequences present in the 60 bp insert I are represented by a heavy bar. The position of the label is indicated by a star. The lengths of the protected fragments are given.

(C) Quantitative S_1 nuclease analysis of cytoplasmic RNA isolated from MCF-7 and HeLa cells cotransfected with 20 µg of pS2MI (lanes, 2,3,6 and 7) or pS2MIE (lanes 4,5,8 and 9) and 1 µg of control plasmid pβ(244+) β, and from untransfected MCF-7 cells (lane 1). Lanes 7 and 9 : the S_1 analysis was performed on 1 to 1 ratio mixed cytoplasmic RNA samples from untransfected MCF-7 cells and from HeLa cells transfected with pS2MI (lane 7) or pS2MIE (lane 9). MCF-7 cells were grown in the presence (+) or absence (-) of oestradiol.

enhancer) encoded transcripts protects a fragment of 104 nt, and that for native pS2 transcripts gives a protected fragment of 150 nt. The probe used for S1 nuclease quantitation of the control pβ(244+)β-encoded transcripts (described in ref. 13) gives rise to a rabbit β globin protected fragment of 60 nt (Glob). Fig. 1C (lanes 1 to 5) shows the results of S1 nuclease mapping analysis from such an experiment [the signal at approx. 72 nt is an artifact arising from contaminating pβ(244+)β plasmid DNA]. It is apparent that, whilst the endogenous pS2 gene is strongly stimulated by exposure of the steroid-withdrawn MCF-7 cells to oestradiol (compare lanes 2 and 3; 4 and 5), the transfected pS2 genes pS2MI (lanes 2 and 3) and pS2MIE (lanes 4 and 5) are expressed at the same level with or without oestradiol. Comparison of the control β-globin signal obtained shows that, in this particular experiment, the efficiency of transfection was much higher in the plates containing pS2MI than in those containing pS2MIE.

HeLa cells do not possess functional oestrogen receptor, nor do they express detectable levels of pS2 mRNA (14). We therefore thought that it would be of interest to determine the relative transcriptional activity of the cloned pS2 gene when introduced into MCF-7 versus HeLa cells in a transient assay. Plasmids pS2MI or pS2MIE were cotransfected with the control pβ(244+)β plasmid into HeLa cells. The results of an S1 nuclease mapping analysis from a typical experiment are shown in Fig. 1C (lanes 6 to 9). It is clear that, although the control globin plasmid is expressed very efficiently in HeLa cells, pS2MI transcripts are barely detectable (lanes 6 and 7). Comparison of the relative signal intensity of pS2MI and globin transcripts obtained for transfected MCF-7 cells (lanes 2,3) with that obtained for transfected HeLa cells (lanes 6 and 7) reveals that the relative expression of pS2MI is at least 10 times higher in the former cell line. The observation that the relative efficiency of transcription of pS2MI is much higher in MCF-7 than in HeLa cells suggests that there may be MCF-7-specific transcriptional factor(s) involved in the expression of the pS2 gene, but which, however, do not appear to be under oestrogen control. Comparison of the relative signal intensity obtained on transfection of HeLa cells with pS2MI (lanes 6 and 7) and pS2MIE (lanes 8 and 9) shows that expression of the transfected pS2 gene is increased to detectable levels by the SV40 enhancer.

In order to investigate whether a potential problem in S1 nuclease quantitation involving titration of pS2MI/pS2MIE-specific probe by excess of endogenous pS2 transcripts was occurring to any significant extent in MCF-7 transfected cells, RNA isolated from untransfected MCF-7 cells (grown in the presence of oestradiol) was added to that of transfected HeLa cells prior to S1 nuclease analysis (Fig. 1C). However, the specific signals from pS2MI and pS2MIE remain unaffected by the excess pS2 transcripts present (compare lane

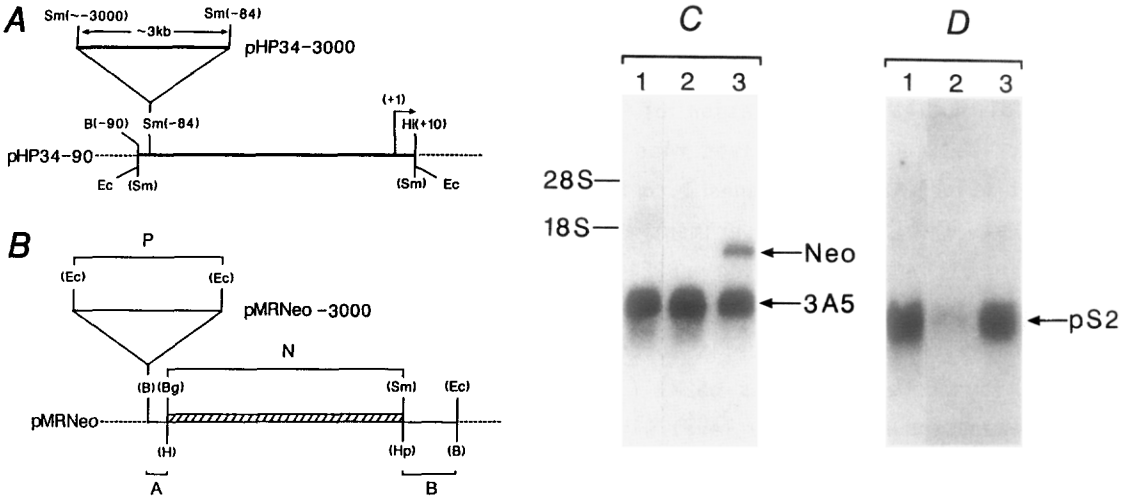

Figure 2. (A) and (B) : Structure of pS2 promoter containing plasmids : pHP34-90 and pHP34-3000 (A) and pMRNeo and pMRNeo-3000 (B). The recombinants are not drawn to scale. Sites in parenthesis were destroyed during plasmid constructions. Positions are given relative to the pS2 capsite (+1). BamHI=B, BglII=Bg, EcoRI=Ec, HindIII=H, HinfI:Hi, HpaI=Hp, SmaI=Sm.
A : SV40 leader sequence ; B : SV40 sequence containing polyadenylation signal and poly A addition site ; P : pS2 3 kb promoter region ; N : Tn5-neo gene.
(C) and (D) : Northern analysis of cytoplasmic RNA isolated from MCF-7 transformants (lanes 2 and 3) containing copies of pMRNeo-3000 stably integrated in the genome and wild type MCF-7 cells (lanes 1). Cells were grown in the presence (lanes 1 and 3) or in the absence (lanes 2) of oestradiol. Neo and 3A5 (panel C) and pS2 (panel D) indicate pMRNeo-3000, 3A5 and pS2 gene encoded transcripts, respectively.

6 with 7 and lane 8 with 9), thus validating the experimental conditions employed.

There are a number of possible explanations as to why transcription of the pS2 gene is not inducible by oestrogen when the gene is introduced into MCF-7 cells in a transient assay. For example, the oestrogen-independent expression may be a consequence of the transient transfection system used to monitor the hormonal regulation of the gene. In order to test this hypothesis, we investigated the oestrogen response of pMRNeo-3000 which was stably integrated into the genome of MCF-7 cells. In pMRNeo-3000, the transcription of the Tn5 neomycin-resistant gene (12) is directed by the pS2 gene promoter region (+10 to approx. -3000) (Fig. 2A and B). Expression of the neo gene renders eukaryotic cells resistant to the drug G-418. The plasmid pMRNeo-3000, linearized with SalI, was introduced into MCF-7 cells by electroporation (15, 16, 17). Twenty four hours after pulsing, cells were diluted five-fold and placed in a selective medium containing 400 µg G-418 sulphate

(GIBCO) and 50 nM oestradiol. G-418-resistant clones appeared after two to three weeks selection at a frequency of approx. 3×10^{-5} clones per cell. Electroporation with control plasmid DNA such as pBR322 or the parent promoterless vector, pMRNeo (Fig. 2), did not give rise to resistant clones at a detectable level. These results suggest that the pS2 promoter region present on pMRNeo-3000 is indeed functional in MCF-7 cells, and is responsible for directing expression of the neo gene. Southern blot analysis of restriction digests of DNA isolated from the stably transformed MCF-7 cell lines revealed that they contained one to several copies of the intact pS2-neo hybrid gene integrated into the genome (data not shown).

In order to investigate the oestrogen response of the hybrid gene in the stable transfectants, heterogenous cell populations of between 50 to 100 G-418-resistant clones were grown in steroid withdrawn medium in the presence or absence of oestradiol. Cytoplasmic RNA was isolated and subjected to Northern analysis (Fig. 2 C and D). The level of "neo-specific" RNA transcripts (panel C) is barely detectable in the absence of oestradiol (lane 2), but rises dramatically in its presence (lane 3). As expected, neo-specific transcripts are not detectable in non-transformed MCF-7 cells (lane 1). The 1.1 kb neo RNA has the predicted size of a transcript initiating at the natural pS2 promoter start site and terminating at the heterologous polyadenylation site of pMRneo-3000. The plasmid 3A5 which is a cDNA clone corresponding to a mRNA species whose level remains unaltered in the presence or absence of oestrogen (7) was used as an internal control in this experiment, in which it is also shown (panel D) that the induction pattern seen for the integrated pS2-neo gene mimics that of the endogenous pS2 gene in the stably transformed MCF-7 cells.

The pools of independent clones analyzed above were plated at low density in withdrawn medium, and subsequently subjected to three different growth conditions : G-418 plus oestradiol (E2), G-418 plus anti-oestrogen tamoxifen (TAM), and tamoxifen only. MCF-7 cells stably transformed with the pAG60 vector carrying neomycin-resistance (12) were treated in an identical fashion, and served as an oestrogen non-responsive control. The subsequent survival of the transformants was then monitored (results summarized in Table 1). These results suggest that the vast majority of clones transformed with pMRNeo-3000 express the neo gene in an oestrogen-dependent manner, the expression being too low to generate resistance to G-418 in the absence of oestrogen. Thus the hormone responsive phenotype appears to be expressed in pMRNeo-3000 transformants of MCF-7 cells.

In summary, modulation of pS2 transcription by oestrogen in MCF-7 cells is controlled by sequences located within the 5'-flanking region of the gene from -3000 to +10, but demonstration of this control is dependent upon

Table 1 : Dependence of the G-418-resistant phenotype of MCF-7 transformants on oestrogen induction of neomycin transcripts (see text for details). 10^{-6} M tamoxifen (TAM), 400 µg/ml G-418 sulphate (GIBCO) (G-418) and 5 x 10^{-8} M 17β-oestradiol (E2) was added to the steroid hormone stripped medium as indicated; (+++) cells reached ≥ 90% confluence in 6 days, (+--) cells reached ≤ 5% confluence in 6 days. The assays were carried out in duplicate and gave similar results.

MCF-7 TRANSFORMED	ADDITION TO THE MEDIUM		
		TAM	E2
	G-418	G-418	
pMRNeo-3000	+++	+--	+++
pAG60	+++	+++	+++

the assay system involved. It is possible that some trans-acting factor(s) required for modulating the oestrogen response may be present in limiting amounts in MCF-7 cells when compared with the amount of potentially responsive DNA which is introduced by calcium phosphate transfection. Under these circumstances, expression of the potentially oestrogen-responsive gene would appear constitutive. Since only a minority of MCF-7 cells are transfected (data not shown), the overall oestrogen regulation of the endogenous pS2 gene would appear essentially normal, as is indeed observed. That the limiting trans-acting factor could be in fact the oestradiol receptor itself is supported by the results of preliminary experiments (A.M. Nunez and M. Berry, unpublished results from our laboratory) which show that expression of a reporter gene containing the -3000 to +10 pS2 promoter region can be induced by oestradiol in transient assays, provided it is co-transfected in HeLa cells with a recombinant that expresses the human oestrogen receptor cDNA (14).

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