Interaction of AP-1-, AP-2-, and Sp1-like Proteins with Two Distinct Sites in the Upstream Regulatory Region of the Plasminogen Activator Inhibitor-1 Gene Mediates the Phorbol 12-Myristate 13-Acetate Response*

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Phorbol 12-myristate 13-acetate induces a 3- and 10-fold induction of chloramphenicol acetyltransferase (CAT) activity in HT1080 and HeLa cells, respectively, following transient transfection of a 336-base pair plasminogen activator inhibitor-1 (PAI-1) promoter fragment linked to a CAT reporter gene. Substitution mutations in the regions encompassing nucleotides -78 to -69 (TGGGTGGGGG) or -81 to -54 (TGAGTTCA), but not in the regions -155 to -149 (TGCCCTCA) or -84 to -76 (AGTGAGTGG) reduced this induction.

Gel electrophoresis of double-stranded -65 to -50 oligonucleotides of the PAI-1 promoter region and nuclear extracts from HeLa cells produced a gel shift pattern similar to that obtained with an AP-1 consensus oligomer, and excess unlabeled AP-1 oligomer reverted binding, suggesting that this region of the PAI-1 promoter is an AP-1-like binding site. Gel electrophoresis of double-stranded -82 to -65 oligonucleotides with HeLa nuclear extracts revealed a gel shift pattern of three bands; Sp1 consensus oligomer competed with the binding to two of these bands and AP-2 consensus sequence oligomer with the binding to the third band. The -82 to -65 oligomer also bound to purified AP-2 and Sp1 proteins. Southwestern blotting of HeLa nuclear extracts revealed that the labeled oligomer spanning region -82 to -65 bound to proteins with molecular masses of 52 and 72 kDa. Consensus AP-2 oligonucleotides competed for binding of the labeled -82 to -65 oligonucleotide to the 52-kDa protein, but consensus Sp1 oligonucleotides did not compete for binding to the 72-kDa compound. The 72-kDa component binding to the -82 to -65 region may represent a new protein involved in transcriptional regulation.

Fibrinolysis is the process in which fibrin, a main component of the blood clot, is degraded by the action of the proteolytic enzyme plasmin. Plasmin is formed through the proteolytic cleavage of the zymogen plasminogen by urokinase-type (u-PA) or tissue-type (t-PA) plasminogen activator (1). The main physiological inhibitor of these plasminogen activators is plasminogen activator inhibitor-1 (PAI-1); increased levels of PAI-1 have been shown to be associated with venous thrombosis (2, 3) and to predispose to arterial thrombosis (4). Plasminogen activators and their inhibitors not only play a pivotal role in maintaining the hemostatic balance in blood but also in other proteolytic processes, such as cell migration and tumor invasion (5, 6). PAI-1 is produced by various cell types, and its synthesis is regulated by several modulators (for references see Ref. 7).

Understanding of the regulation of the PAI-1 gene expression may open perspectives for the pharmacological modulation of PAI-1 levels in patients with thromboembolic disease. Previous studies have shown that DNA fragments spanning base pairs -791 to -546 and -328 to -186 of the PAI-1 promoter region are involved in the regulation by transforming growth factor-β (8) and that a fragment spanning nucleotides -805 to +72 (9) or -305 to +75 (10) confers glucocorticoid responsiveness to a reporter gene. Recently, we have shown that a 826-bp (-806 to +18) PAI-1 promoter fragment linked to a CAT reporter gene provides responsiveness to induction with phorbol 12-myristate 13-acetate (PMA) when transfected in HeLa, HT1080, and human umbilical vein endothelial (HUVEC) cells (11), the extent of induction being similar to that of the endogenous PAI-1 antigen. A truncated 336-bp PAI-1 promoter fragment containing nucleotide -318 to +18 had a similar responsiveness to PMA induction, suggesting that PMA-responsive sequences are present in the first 336 bp (11). Since the PMA responsiveness of several genes is mediated via AP-1, AP-2, or NF-κB binding sites (12–17), we studied the effects of mutagenesis of three AP-1-like and one AP-2-like binding sites in this 336-bp PAI-1 promoter fragment on its responsiveness to PMA induction. Two PMA-responsive sequences were identified, and an effort was made to identify specific nuclear factors binding to these elements.

**Materials and Methods**

**Reagents**—HT1080 cells and Hep3B were obtained from the American Type Culture Collection, the HeLa cell line was provided by Dr. *The abbreviations used are: u-PA, urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; bp, base pair(s); CAT, chloramphenicol acetyltransferase; PMA, phorbol 12-myristate 13-acetate; DME, Dulbecco's modified Eagle's medium; MEM, modified Eagle's medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CRE, CAMP-responsive element; CREB, CRE binding protein.

1. Received for publication, December 16, 1991.
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The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories, Brussels) supplemented with streptomycin (100 µg/ml), penicillin (100 IU/ml), glutamine (1 mM), sodium bicarbonate (2 mM), MEM nonessential amino acids (1×), and 10% fetal calf serum (Life Technologies, Ghent, Belgium).

Endoproteinase Glu-C from Staphylococcus aureus V8 was obtained from Boehringer Mannheim. Purified c-Jun, Sp1, and AP-2 protein were purchased from Promega (Madison, WI). Oligonucleotides were synthesized using a cyclone synthesizer (Millipore, El Paso, TX) according to the specifications of the manufacturer.

Plasmids—Two fragments (826 and 336 bp, respectively) from the recombinant plasmid pECEG23.7 (18) carrying a genomic PAI-1 clone were inserted into the promoterless CAT vector pOCAT2 (19). The fragments had identical 3'-ends (i.e., the PstI site at position +18) and different 5'-ends. The relevant PAI-1 promoter fragments were provided with SalI linkers and cloned into the pOCAT2 vector, in which the unique BamHI site was modified to a SalI site.

Transfection Experiments and Chloramphenicol Acetyltransferase (CAT) Assays—Cells were seeded at a density of 2×10^4 cells/cm^2 into 75-cm^2 Petri dishes and cultured 18 h before transfection was performed using the calcium phosphate method (20). Three and one-half hours after the addition of the precipitate to the cells, a 45-s glycerol shock was carried out with 15% glycerol in phosphate-buffered saline after which the cells were washed twice with phosphate-buffered saline and allowed to recover in supplemented DMEM. Six hours later the medium was replaced with fresh serum-free DMEM containing 160 nM PMA or solvent. After 12-h incubation, the cells were harvested by scraping from the dish and were disrupted by a repeated freeze/thaw cycle. The CAT assay was performed according to Neuman et al. (21). Prior to the addition of the labeled butyryl-CoA, the extract was heated to 60 °C in the presence of 5 mM EDTA, according to Crab et al. (22). For each sample the production rate of [14C]butyryl-chloramphenicol was measured during the first 6 h.

Site-directed Mutagenesis—Base substitutions were made by oligonucleotide-mediated mutagenesis (23) in four different regions identified as box A, box B, box C, and box D, corresponding to nucleotides -61 to -54, -78 to -69, -84 to -76, and -155 to -149, respectively. The mutant oligonucleotides used as primers are listed in Table I together with the corresponding wild-type PAI-1 sequences.

Preparation of Nuclear Extracts—Cells were stimulated for 12 h with 160 nM PMA before the harvest. Nuclear extracts were prepared essentially as described by Ohlsson et al. (24) except that the buffers contained 1 mM EDTA and that nuclei were disrupted by the addition of 0.42 M NaCl.

Gel Shift Experiments—The double-stranded deoxyoligonucleotides used in the gel shift experiments are listed in Table I. Deoxyoligonucleotides were labeled either by using DNA polymerase I Klenow fragment and [γ-32P]dCTP or by end-labeling using T4 kinase.

Addition of 160 nM PMA to HT1080 or HeLa cells transfected with a construct containing the wild-type 336-bp PAI-1 promoter fragment induced the CAT activity 3- and 10-fold in HT1080 and HeLa cells, respectively. Similarly CAT activity was induced 2.5-fold in the human liver cell line Hep3B. Mutations in the box C (nucleotides -84 to -76) or in the box D (nucleotides -155 to -149) had no effect on the PMA induction, whereas mutations in the box A (nucleotides -61 to -54) or in the box B (nucleotides -78 to -69) inhibited PMA induction in HT1080 cells virtually completely and in HeLa cells by approximately 50 percent. Double mutants with substitutions in two of the four boxes were therefore constructed and transfected. When HT1080 cells were transfected with double mutants, no PMA induction was obtained when one of the mutations was localized in the box A or box B (results not shown). In HeLa cells, PMA induction was markedly reduced with double mutants in which one of the mutations was localized in either box A or box B (Fig. 2). Furthermore, double mutants in box A and box B had virtually lost all the PMA responsiveness, whereas double mutants of box C and box D had a responsiveness similar to that of the wild-type promoter.

RESULTS

Identification of PAI-1 Promoter Sequences (Nucleotides -61 to -54 and -78 to -69)—A summary of the results of PMA induction of CAT activity in HT1080 (Fig. 1A) and HeLa (Fig. 1B) cells transfected with the 336-bp wild-type and mutant PAI-1 promoter fragments (see Table I) fused to the CAT reporter gene. Addition of 160 nM PMA to HT1080 or HeLa cells transfected with a construct containing the wild-type 336-bp PAI-1 promoter fragment induced the CAT activity 3- and 10-fold in HT1080 and HeLa cells, respectively. Similarly CAT activity was induced 2.5-fold in the human liver cell line Hep3B. Mutations in the box C (nucleotides -84 to -76) or in the box D (nucleotides -155 to -149) had no effect on the PMA induction, whereas mutations in the box A (nucleotides -61 to -54) or in the box B (nucleotides -78 to -69) inhibited PMA induction in HT1080 cells virtually completely and in HeLa cells by approximately 50 percent. Double mutants with substitutions in two of the four boxes were therefore constructed and transfected. When HT1080 cells were transfected with double mutants, no PMA induction was obtained when one of the mutations was localized in the box A or box B (results not shown). In HeLa cells, PMA induction was markedly reduced with double mutants in which one of the mutations was localized in either box A or box B (Fig. 2). Furthermore, double mutants in box A and box B had virtually lost all the PMA responsiveness, whereas double mutants of box C and box D had a responsiveness similar to that of the wild-type promoter.

Binding of Nuclear Factors to Box A (Nucleotides -65 to -50)—To identify proteins binding to the box A oligomer...
Deoxyoligonucleotides used for mutagenesis and gel shift experiments

| Identification                | Nucleotide sequence                  | Mutant PAI-1* |
|------------------------------|--------------------------------------|--------------|
| A. Deoxyoligonucleotides used for mutagenesis |                        |              |
| -71 to -48 region            | GGCTGGACATGAGTTCATCTATT              | Mutant A     |
| -86 to -63 region            | CCAATGATGGGTTTGGGCGCTGAGC           | Mutant B     |
| -91 to -70 region            | CAGAGCCAGTGTGGGTCGGGG               | Mutant C     |
| -165 to -140 region          | CACACACACATGCTGACATCTGCC            | Mutant D     |

* Nucleotide sequences identical to those in wild-type PAI-1 are indicated with dashes. The mutants were designed to introduce a HindIII restriction site in mutants A and D and a EcoRI site in mutant B to allow identification by restriction digestion.

**Fig. 2.** PMA induction of CAT activity in HeLa transfected with PAI-1 promoter/CAT reporter gene constructs. The PAI-1 promoter fragment consisted of the 336-bp wild-type (ut.) sequence or of double mutants in the A, B, C, or D boxes as described in Table I. --, without PMA stimulation (filled bars); +, after stimulation with 160 nM PMA (hatched bars); blank, background CAT activity in untransfected cells. The data represent results of one of three qualitatively comparable experiments, each performed in duplicate. The results are expressed in absolute values (Δ counts/h).

**Fig. 3.** Gel shift experiments with labeled double-stranded oligomer covering box A (nucleotides -65 to -49). A, labeled PAI-1 box A oligonucleotide incubated with HeLa nuclear extract. Lane 1, unstimulated cells; lane 2, PMA-treated cells. B, labeled PAI-1 box A oligonucleotide incubated with nuclear extracts from PMA-stimulated HeLa cells and increasing amounts of unlabeled competitor DNA (cf. Table I). Lane 1, no competitive oligonucleotides added; lanes 2–4, 10, 25, or 50 ng of consensus CRE oligonucleotide added, respectively; lanes 5–7, 10, 25, or 50 ng of consensus AP-1 oligonucleotide added, respectively; lane 8, 50 ng of consensus AP-2 oligonucleotide added; lane 9, labeled consensus AP-1 oligonucleotide incubated with HeLa nuclear extracts C, labeled oligomer covering box A incubated with purified c-Jun protein and competitor DNA. Lane 1, no competitive DNA added; lanes 2 and 3, 10 and 25 ng of consensus AP-1 oligonucleotide added, respectively; lane 4, 25 ng of consensus CRE oligonucleotide added; lane 5, 25 ng of consensus AP-2 oligonucleotide added.

containing nucleotides -65 to -50, gel shift experiments were performed. Nuclear extracts obtained from HeLa cells contained proteins which bound to the box A sequence as shown by the presence of DNA-protein complexes with altered mobility (Fig. 3A). Nuclear extracts from PMA-treated HeLa cells (lane 2) revealed enhanced binding to the box A oligonucleotide as compared with extracts from untreated cells (lane 1), suggesting that enhanced binding of nuclear proteins to the box A might be implicated in the transcriptional response to PMA induction. The specificity of the complexes was demonstrated by competition experiments with unlabeled oligonucleotides (Fig. 3B), representing the consensus binding sites for the transcription factors AP-1, AP-2, and CRE as defined in Table I. Competition with the binding of nuclear factors to the box A oligomer was observed with CRE consensus oligonucleotides (lanes 2–4) and with AP-1 consensus oligonucleotides (lanes 5–7), whereas the AP-2 consensus oligonucleotide (lane 8) did not compete (Fig. 3B).

The specificity of the box A oligomer for AP-1-like proteins was confirmed by its binding to purified c-Jun protein (Fig. 3C). Effective competition with this binding was also obtained with cold consensus AP-1 (lanes 2 and 3) and CRE (lane 4) oligonucleotides, but not with cold consensus AP-2 oligonucleotide (lane 5). Partial digestion of nuclear extracts with
endoproteinase Glu-C resulted in similar changes in the gel shift patterns obtained with the box A oligomer and the AP-1 consensus oligomer (Fig. 4), confirming the similarity or identity of the nuclear protein reacting with these oligomers.

Binding of Nuclear Factors to Box B (Nucleotides −82 to −65)—Gel shift experiments of nuclear extracts of PMA-stimulated HeLa cells and labeled box B oligomer covering nucleotides −82 to −65 revealed several bands with reduced mobility, marked as bands 1, 2, and 3 (Fig. 5A). Excess of unlabeled consensus Sp1 oligonucleotide (lanes 2–4) reduced the binding of bands 2 and 3 to the labeled box B oligomer, whereas unlabeled consensus AP-2 oligonucleotide (lanes 5–7) competed for the binding of band 1 components, suggesting that both AP-2- and Sp1-like proteins are able to bind to this region.

Gel shift experiments with purified DNA binding proteins and labeled box B oligomer (Fig. 5B) revealed binding of purified AP-2 (lane 1) and Sp1 (lane 2) protein, but not of purified c-Jun protein (not shown). Binding of purified Sp1 factor to the B box was enhanced in the presence of HeLa nuclear extract (Fig. 5C). The presence of Sp1 protein yielded a retarded band which did not correspond to one of the three bands obtained with extract only. PMA stimulation of HeLa cells produced enhanced binding of nuclear extracts to the labeled box B oligomer (Fig. 5D). Especially the binding of band 1 was enhanced. This again suggests that enhanced binding of nuclear factors to the region spanning nucleotides −82 to −65 in PAI-1 might be involved in the transcriptional response to PMA.

In order to directly identify proteins present in the box B complexes, Southwestern blotting experiments were performed. Nuclear extracts from PMA-stimulated HeLa cells were separated by SDS-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose, and the filter was probed with radioactive-labeled oligonucleotides (24). Southwestern blotting of HeLa nuclear extracts with labeled box B oligonucleotide revealed its binding to proteins with molecular masses of 52 and 72 kDa (Fig. 6A). A labeled PAI-1 box B oligonucleotide was incubated with PMA-stimulated HeLa nuclear extracts and with increasing amounts of unlabeled competitor DNA (cf. Table I). Lane 1, no competitive oligonucleotide added; lanes 2–4, 10, 25, or 50 ng of consensus Sp1 oligonucleotide added, respectively; lanes 5–7, 10, 25, or 50 ng of consensus AP-2 oligonucleotide added, respectively; lane 8, 50 ng of consensus AP-2 and 50 ng of Sp1 oligonucleotide added. B, labeled box B oligonucleotide incubated with purified AP-2 protein (lane 1) or with purified Sp1 protein (lane 2). C, labeled PAI-1 box B oligonucleotide incubated with nuclear extracts from HeLa cells (lane 1) with nuclear extracts from HeLa cells and purified Sp1 protein (lane 2) or with purified Sp1 protein alone (lane 3). D, labeled PAI-1 box B oligonucleotide incubated with nuclear extracts from HeLa cells without (lane 1) or with (lane 2) stimulation with PMA.

FIG. 5. Gel shift experiments with labeled double-stranded oligomer covering box B (nucleotides −82 to −61). A, labeled PAI-1 box B oligonucleotide was incubated with PMA-stimulated HeLa nuclear extracts and with increasing amounts of unlabeled competitor DNA (cf. Table I). Lane 1, no competitive oligonucleotide added; lanes 2–4, 10, 25, or 50 ng of consensus Sp1 oligonucleotide added, respectively; lanes 5–7, 10, 25, or 50 ng of consensus AP-2 oligonucleotide added, respectively; lane 8, 50 ng of consensus AP-2 and 50 ng of Sp1 oligonucleotide added. B, labeled box B oligonucleotide incubated with purified AP-2 protein (lane 1) or with purified Sp1 protein (lane 2). C, labeled PAI-1 box B oligonucleotide incubated with nuclear extracts from HeLa cells (lane 1) with nuclear extracts from HeLa cells and purified Sp1 protein (lane 2) or with purified Sp1 protein alone (lane 3). D, labeled PAI-1 box B oligonucleotide incubated with nuclear extracts from HeLa cells without (lane 1) or with (lane 2) stimulation with PMA.

FIG. 6. Southwestern blotting of nuclear extracts of PMA-stimulated HeLa cells, treated with labeled box B oligonucleotide (A), with labeled AP-2 consensus oligonucleotide (B), or with labeled Sp1 consensus oligonucleotide (C).

FIG. 7. Southwestern blotting of nuclear extracts of PMA-stimulated HeLa cells treated with labeled box B oligonucleotide and competitor DNA. A, no competitive DNA added. B, 250 ng of box B oligonucleotide added. C, 250 ng of consensus AP-2 oligonucleotide added. D, 250 ng of consensus Sp1 oligonucleotide added.

In order to directly identify proteins present in the box B complexes, Southwestern blotting experiments were performed. Nuclear extracts from PMA-stimulated HeLa cells were separated by SDS-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose, and the filter was probed with radioactive-labeled oligonucleotides (24). Southwestern blotting of HeLa nuclear extracts with labeled box B oligonucleotide revealed its binding to proteins with molecular masses of 52 and 72 kDa (Fig. 6A). A labeled consenus AP-2 oligomer also bound to a 52-kDa component in the nuclear extracts from HeLa cells (Fig. 6B), suggesting that the oligomer covering box B and the AP-2 consensus oligomer bind to the same protein or to two proteins with similar molecular mass. Consensus Sp1 oligonucleotide (Fig. 6C) also recognized...
a 72-kDa component, in addition to other components with higher and lower molecular weight. Unlabeled B box oligonucleotides competed for binding of labeled B box oligonucleotide to both the 52- and 72-kDa factors (Fig. 7B), whereas AP-2 oligonucleotides specifically competed for the binding to the 52-kDa protein (Fig. 7C). However, no competition was obtained with unlabeled consensus Sp1 oligonucleotides (Fig. 7D).

DISCUSSION

PMA induces CAT activity in HT1080 and HeLa cells transfected with an 826-bp or with a 336-bp PAI-1 promoter fragment linked to a CAT reporter gene 3' and 5'-flank, respectively (11). When these constructs were transfected in the human liver cell line Hep3B, both produced a 2.5-fold induction of CAT activity. PMA induces a similar extent of induction of endogenous PAI-1 antigen levels in all these cells. In order to identify PMA-responsive sequences in the first 336 bp of the PAI-1 promoter region, we have mutated three potential AP-1-like and one AP-2-like sequences, corresponding to nucleotides −155 to −149 (box D), −84 to −76 (box C), −78 to −69 (box B), and −61 to −54 (box A), respectively. Mutations made in the boxes D and C had no effect on the PMA response, whereas mutations in the boxes A and B caused a complete block of the PMA induction in HT1080 cells and a partial inhibition in HeLa cells.

Gel shift experiments using nuclear extracts from HeLa cells and labeled double-stranded oligomers representing boxes A and B revealed labeled DNA-protein complexes. Moreover, enhanced binding was obtained with nuclear extracts prepared from HeLa cells pretreated with PMA. Interestingly, the region spanning nucleotides −79 to −48, which contains these two binding sites, is identical with the corresponding region in the rat PAI-1 gene (26). The interspecies conservation of these PMA-responsive sequences suggests that they may constitute binding sites for important cis-acting elements.

Several approaches were used to identify proteins involved in the binding to these boxes A and B. Gel shift experiments with oligonucleotides representing the AP-1 consensus sequence showed the same pattern of DNA-protein complexes as obtained with the box A oligomer, and partial digestion of the nuclear extract with endoproteinase Glu-C resulted in the conservation of these PMA-responsive sequences suggesting that they may contribute binding sites for important cis-acting elements.

The labeled oligomer covering box B produced a rather complex gel shift pattern with nuclear extracts from PMA-stimulated HeLa cells, suggestive of the involvement of several proteins. Competition experiments with AP-2 consensus oligomers demonstrated competition for nuclear factors binding to the oligomer covering box B. Gel shift experiments also revealed that the Sp1 consensus sequence competed for binding to the labeled box B oligomer. Moreover, purified AP-2 and Sp1 protein were found to bind to labeled box oligomer. Southwestern blots with labeled consensus Sp1 oligonucleotide revealed a 72-kDa component in addition to several other bands, whereas probing with consensus AP-2 oligonucleotide only revealed a 52-kDa component. The 72-kDa component did not appear to be a proteolytic degradation product of the Sp1 protein, since it was consistently observed in all HeLa cell nuclear extracts as well as in nuclear extracts from HT1080 cells and from rat liver (data not shown).

Furthermore, excess consensus Sp1 oligonucleotides did not compete for binding of the labeled box oligonucleotide to the 72-kDa protein, which argues against the hypothesis that the 72-kDa protein represents a degradation product of the Sp1 protein and that the 72-kDa protein identified in the present study corresponds to any of the known Sp1-like proteins (35-38). Furthermore, no DNA binding protein with a mass of 72-kDa has been reported which recognizes sequences similar to those of the box B oligomer, suggesting that this 72-kDa component may be a new transcription factor. However, at least seven different proteins have been identified in nuclear extracts from A204 cells which react with monoclonal antibodies raised against the zinc finger domain of Sp1, two of which have molecular masses in the 70-75-kDa range (39). It is possible that the present 72-kDa component may represent one of these proteins. The presence of both AP-2- and Sp1-like proteins, which are both GC box binding proteins, in the gel shift complexes suggests that they may bind in a juxtaposed manner to the GGTTGGGGCTGGAC sequence in the oligomer covering box B. Such a binding had already been suggested for 21-bp repeats of the SV40 early promoter (16).

In conclusion we have identified two sequences in the PAI-1 promoter region, identified as the boxes A and B, which are involved in the trans-response of the PAI-1 gene to PMA. Mutations in each of these boxes have an inhibiting effect on the PMA induction of the PAI-1 promoter in HT1080 and HeLa cells. The box A contains sequences related to the consensus AP-1 binding site, and in this study we have shown that AP-1-like proteins are able to bind to that region. The box B sequence is related to the consensus AP-2 binding site, and we have demonstrated that both AP-2 and a probably hitherto unidentified 72-kDa protein can bind to this region.

Acknowledgment—We are grateful to V. Feytons, Division of Biochemistry of the Faculty of Medicine, University of Leuven, Belgium for the oligonucleotide synthesis.

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