A simple and sensitive method for determining transcription initiation site: identification of two transcription initiation sites in rat group II phospholipase A_2 gene

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ABSTRACT

We developed a simple and sensitive method for assigning transcriptional initiation sites, and applied it to characterize the transcriptional unit of rat group II phospholipase A_2 (PLA_2) gene. Our method involves the primer extension reaction followed by detection of its products by hybridization. Using this method, we were able to map two transcriptional initiation sites on the nucleotide sequence of the core promoter region of PLA_2 gene with one-base resolution without any difficulties.

INTRODUCTION

Mammalian phospholipases A_2 characterized so far have been classified into two groups on the basis of their characteristics in the primary structure: group I enzymes are abundantly present in the pancreas as well as in lung, gastric mucosa and spleen (1–3), while group II enzymes have been isolated from various sources including platelets, spleen, intestine, ascites, peritoneal exudate, and rheumatoid arthritic synovial fluid (4–9). We have recently reported that rat aortic smooth muscle cells (SMCs) secrete a large amount of group II phospholipase A_2 (PLA_2) when stimulated with cAMP-elevating agents and inflammation-related factors such as tumor necrosis factor-alpha (TNF), interleukin 1-beta (IL-1), and lipopolysaccharide (LPS) (10). Since PLA_2 is found in some inflammatory regions and induces inflammatory responses when injected into animals (11,12), this enzyme may play some role in the progression of some vascular disorders related to inflammation. In this context, we think it important to understand the regulation mechanism of PLA_2 gene expression in SMCs. As the first step toward this end, we isolated the core promoter region of this gene using a single-sided polymerase chain reaction (PCR) assisted by adaptor ligation (13), and assigned the transcription initiation sites on the nucleotide sequence thus obtained. For mapping of the transcription initiation sites, we combined a conventional primer extension technique (14) with a genomic sequencing method (15) to improve both the sensitivity and the specificity and to simplify the overall procedure. This improvement enabled us to detect even a minor transcription initiation site as well as a major one on PLA_2 gene without any difficulty.

MATERIALS AND METHODS

Culture and treatment of SMCs

SMCs were isolated by enzymatic digestion of media of thoracic aorta from male Sprague–Dawley rats as described by Chamley-Campbell *et al.* (16). The cells were cultured in Dulbecco's modified Eagle's medium containing 20% fetal calf serum. For stimulation with factors, confluent SMCs were washed twice with the medium specified above including 0.1 mg/ml bovine serum albumin in place of 20% fetal calf serum. After washing, SMCs were incubated for 24 hr in a medium with the factor to be examined (or a combination of factors), and then analyzed.

RNA blotting analysis

Total cellular RNA was extracted from SMCs as described by Chomczynski and Sacchi (17). RNA (15 μg) was run on 1% agarose/2.2 M formaldehyde gel, and then transferred onto a nylon membrane (Biodyne A, Nihon Pall, Ltd.) according to standard procedures (18). After baking and UV-crosslinking, reversible RNA staining with methylene blue was performed to check the amount and quality of RNA (19). After destaining, the membrane was subjected to hybridization under the conditions described by Church and Gilbert (15). Rat PLA_2 cDNA (20) was labeled with [alpha-^{32}P]dCTP (New England Nuclear, 3000 Ci/mmole) using a random primer labeling system (Amersham International plc.), and used as a probe for hybridization. After hybridization, the membrane was washed with 15 mM NaCl/1.5 mM sodium citrate (pH 7.0)/0.1% sodium dodecylsulfate at 65°C several times. The signal was detected by autoradiography using Kodak X-OMAT X-ray film with intensifying screens (Cronex Lightning Plus, DuPont) at −70°C for 18 hr.

Primer extension reaction followed by hybridization-based detection of the products

Using total cellular RNA or poly $(A)^+$ RNA, we synthesized a cDNA with oligonucleotide primer 1 (listed in Table 1; its binding site is illustrated in Fig. 2) under the following conditions: 20–160 μg of total cellular RNA or 2 μg of poly $(A)^+$ RNA, 50 pmoles of primer 1, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 250 μM 4dNTPs, 50 u RNase Inhibitor (Takara Shuzo, Co.), 200 u Moloney murine leukemia virus reverse transcriptase (Bethesda Research

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Table 1. Nucleotide Sequences of Oligonucleotides Used in This Study.

Oligomer No.	Sequence (5′−3′)	Binding site[1]
1	ACATCAGCTCTCTTTCCTGT	176
2	ATGAAGGTCCTCCTGTTGCTAGCAG	58
3	ATGGTGGTATGGCTTCTTGT	35
4	CTTGTTCAAGGGCCTGCCTT	20
5	CAGCCCTGCAGAGGGAAGAGC	−52
6	GTTGTCCTCCTGGAAGTA	75
A	TAATACGACTCCGAATTCGG	
B	CCGAATTCGG	

[1] The numbers indicate the 5′-end binding positions of oligonucleotides as shown in Fig. 2.

Laboratories), at 37°C for 1 hr. Before addition of reverse transcriptase, primer 1 and template RNA were allowed to anneal for 15 min at 37°C. After reverse transcription, the cDNA was recovered by ethanol precipitation with 5 μg of sonicated calf thymus DNA. The precipitate was dissolved in 40 μl of 0.3 M NaOH/30 mM ethylenediamine tetraacetic acid, and then boiled for 5 min. After boiling, 10 μl of 1 M Tris-HCl (pH 7.5) was added to neutralize the solution, and then the cDNA was ethanol precipitated in the presence of 2.5 M ammonium acetate. The recovered DNA was dissolved in formamide/xylene cyanol/bromophenol blue solution (3 μl), and then loaded on a 6% polyacrylamide/7 M urea gel (0.4-mm thick) as described for DNA sequencing (21). Using a Sequenase dideoxy sequencing kit from United States Biochemical Co. with primer 1 and PLA_2 cDNA (cloned into Sma I−EcoRI site in pGEM3, Promega Biochem.), a size marker was prepared and run on the same gel. After the electrophoresis, the separated DNA was electrotransferred onto a nylon membrane, and then crosslinked by UV irradiation as described elsewhere (15). The resultant membrane was subjected to hybridization with oligonucleotide probe 2 labeled by tailing with [alpha-^{32}P]dATP (New England Nuclear, 6000 Ci/mmole) (22) under the conditions described by Church and Kieffer-Higgins (23). After hybridization, the membrane was washed several times with 150 mM NaCl/15 mM sodium citrate (pH 7.0)/0.1% sodium dodecylsulfate at 40°C for 15 min. The signal was detected by autoradiography with Kodak X-OMAT or Fuji RX film at room temperature or at −70°C with intensifying screens.

Isolation of the 5′-flanking region of rat PLA_2 gene

According to the method of Mueller and Wold, DNA fragment including the 5′-flanking region of the PLA_2 gene was obtained by single-sided PCR assisted by adaptor ligation (13). Rat genomic DNA was prepared by a standard method (24) and completely digested with Hind III. The digested genomic DNA was denatured by boiling, and then used as a template for primer extension with primer 3 and sequenase. After primer extension, the resultant DNA was ligated with an adaptor (primers A and B, see Table 1). The adaptor ligated DNA was utilized for two successive rounds of PCR: the first with primers A and 3, and the second with primers A and 4. PCR conditions were as described previously (25), and each PCR round consisted of 20 cycles. The oligomers used for the amplification (two oligomers to form the adaptor, A and B; PLA_2 gene specific primers, 3 and 4) are listed in Table 1, and the annealing sites of primers 3 and 4 are shown in Fig. 2. The amplified DNA of the expected size (about 500 base pairs (bp)) was directly sequenced by the method of Ohara *et al.*(25).

Miscellaneous procedures

PCR was performed with genomic or cDNA templates using a Perkin Elmer Cetus DNA Thermal Cycler as described previously (25). Oligonucleotides were synthesized on a Pharmacia Gene Assembler Plus DNA synthesizer. Although the oligomers for PCR were cleaned up only by repeated ethanol precipitation, the others were purified on 20% polyacrylamide/7 M urea gels. cDNA templates for PCR were prepared from total cellular RNA with Moloney murine leukemia virus reverse transcriptase as described in literature (25).

Materials

Human recombinant TNF was obtained from Genzyme. The amount of TNF was expressed in units as defined by the supplier. LPS from *Escherichia coli* was purchased from Pasel.

RESULTS

Isolation of 5′-flanking region of rat PLA_2 gene

We utilized adaptor ligation-assisted PCR to obtain the 5′-flanking region of the PLA_2 gene (13). In a preliminary experiment, we explored for restriction sites located close to the 5′-most sequence of PLA_2 cDNA (20) by genomic DNA blotting analysis, and found that the Hind III site is present at about 500 bp upstream of the 5′-most sequence. Rat genomic DNA (from Sprague−Dawley rats), therefore, was completely digested with Hind III and subject to the single-sided PCR assisted by adaptor ligation. The first PCR cycles were done with primers A and 3, and the second one with primers A and 4. The use of the nested primers for PCR improved both the specificity and the degree of amplification. After two successive PCR rounds, DNA of the expected size (about 500 bp) appeared on the ethidium bromide-stained agarose gel (Fig. 1). The amplified DNA was recovered from the gel, and then directly sequenced without subcloning (25). Figure 2 shows the nucleotide sequence of the amplified DNA together with the downstream cDNA sequence previously reported (20). To eliminate the possibility of PCR artifacts, we tried to amplify PLA_2 gene with two primers, primer 5 (designed from the obtained sequence) and primer 3 (designed from the cDNA sequence reported previously), and found that the DNA of the expected size had been successfully amplified from genomic DNA template (data not shown). We therefore concluded that the obtained fragment is actually located upstream of the 5′-most cDNA sequence.

Effects of various agents on the levels of PLA_2 mRNA in SMCs

Before analyzing the transcription initiation sites, we examined the PLA_2 mRNA levels in SMCs with or without stimuli (Fig.

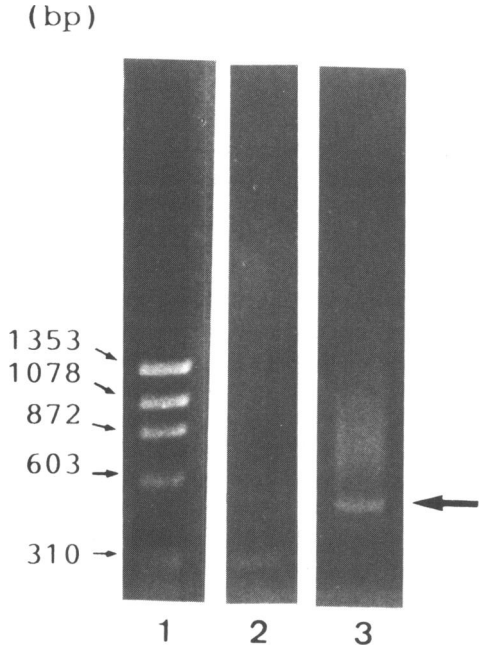

Figure 1. Detection of the products of PCR assisted by adaptor ligation. Aliquots (10 μl) of 100-μl PCR reaction mixture were analyzed on 1% agarose gel. After two successive rounds of PCR, the DNA band of the expected size (about 500 bp) appeared on the ethidium bromide-stained gel (indicated by the arrow). Lane 1, phiX DNA digested with Hae III (size marker); lane 2, first round of PCR with primers A and 3; lane 3, second round of PCR with primers A and 4.

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-497 AAGCTTCTGTGATGGAATGAATGACTGACACGTGAATTAAGCAGCGTACA -448
-447 GAACCAGCGTTCCCTTTCCTATCCCCCAAAGTCACAGTTTATCAGAAGAA -398
-397 AGAAACATCCCAGACTTTCAAACACTCATTCCCTCCCCTGTATACAGTCC -348
-347 TTGCATGCTCCTGGAAGTGGCTGCCAGGAGGCTGCAGGCGGTCACACCCA -298
-297 GATGGGCTTTTGGAAAGTTCTCCAGTCAGGAGCTGCACCCTGTTTCTCAT -248
-247 CAACCGAATGAACTTTCGAAATCAGCTAAAGTTTATGATGGCCACAACCC -198
-197 ATGGTATGAGGGCTTTTCCGCCCTCAAGGCTGTTCTGCCAGCTGTTGGGG -148

-147 GGAAAAGGGGAAATTACCCAGGGCGTTGGGTATGCCCGTCTGTGAATCCA -98
 -97 TTATTTGGCCACACCCACCTCCCCATCCCTGTGCTCTCCGATCCCCAGCC -48

 -47 CTGCAGAGGGAAGAGCTATTTAAGAGCATTGGGAGTACAGGAAAAACaag   3
       5
   4 gcaggcccttgaacaagaagccataccaccatcccatccaagagagctga  53
       4       3
  54 cagcatgaaggtcctcctgttgctagcagttgtgatcatggcctttggct 103
           6       2
 104 caattcaggtccaggggagccttctggagtttgggcaaatgattctgttt 153

 154 aagacaggaaagagagctgatgttagctatggcttctacggttgccattg 203
               1
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Figure 2. The nucleotide sequence of the 5′-flanking region of PLA_2 gene. The nucleotide sequence of the obtained 5′-flanking region (referred to as the 500-bp fragment, the actual size is 497 bp) is shown by upper-case letters, together with the downstream cDNA sequence indicated by lower-case letters. The horizontal arrows indicate the binding sites and the direction of the primers listed in Table 1. The pointed end of the arrows shows the 3′- end. Vertical arrows indicate the transcriptional initiation sites determined in this study, which come from the data shown in Fig. 5. Closed triangles point out the position of the introns of this gene.

3). The stimulants examined here are as follows: TNF, forskolin (cAMP-elevating agent), LPS, cycloheximide (CHX, an inhibitor of protein synthesis) and a combination of some of these agents. As reported previously (10), TNF and forskolin elevated PLA_2 mRNA levels in SMCs. Furthermore, the RNA blotting analysis revealed the following points:(1) CHX alone raised the PLA_2

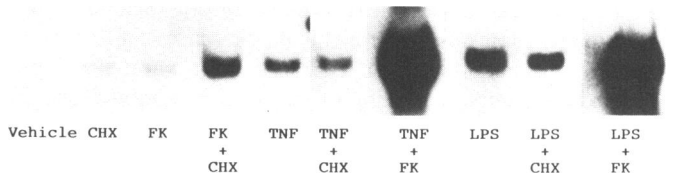

Figure 3. RNA blotting analysis of SMCs treated with and without various stimuli. RNAs isolated from SMCs with various stimuli were analyzed by RNA blotting. The constant amount of total RNA (10 μg) was loaded on a formaldehyde/agarose gel. In this figure, forskolin is abbreviated as FK. The concentrations of stimulants are as follows: forskolin (FK), 10 μM; CHX, 10 μg/ml; TNF, 200 u/ml; LPS, 100 ng/ml.

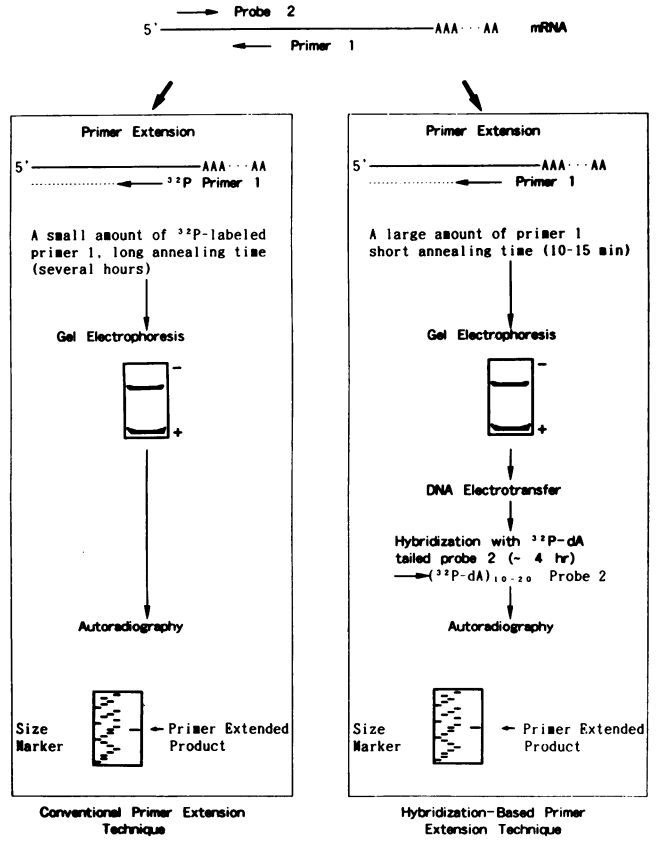

Figure 4. Schematic drawing of the procedures of our hybridization-based primer extension method. These are the steps involved in our primer extension method and the conventional one.

mRNA levels in SMCs, in the order of LPS > TNF > forskolin = CHX under the conditions employed here;(2) the combination of stimuli (forskolin and TNF, forskolin and LPS, or forskolin and CHX) synergistically increased the amounts of PLA_2 mRNA;(3) TNF- and LPS-induced elevation of PLA_2 mRNA levels was not affected significantly by CHX. CHX had no effect on intracellular cAMP concentrations at all (data not shown).

Detection of transcription initiation sites in PLA_2 gene

Next, we tried to assign the transcription initiation site(s) of PLA_2 gene to its nucleotide sequence. To obtain this information, we developed a new technique based on the primer extension reaction. Figure 4 outlines our method for comparison with the conventional oligonucleotide primer extension technique

for mapping transcriptional start sites. In our method, primer extension reaction was performed in the presence of a large molar excess of primer 1 over PLA_2 transcript to shorten the time required for complete binding of the primer. Although specific priming of cDNA synthesis could not be expected to occur under these conditions, the spurious primer extension products are not visualized in our method. This situation is quite different from the case of a conventional primer extension method; high specificity in the detection of the PLA_2 transcription initiation site can be achieved by stringent hybridization with the probe 2 which specifically binds to PLA_2 cDNA in our method. Using this technique, we found that there were two initiation sites in the PLA_2 gene, the downstream one being the major site and the upstream one the minor site (Fig. 5). Figure 5 showed that some primer extended products were one or two bases shorter than full length, suggesting that methylation of the ultimate or penultimate residue of the PLA_2 transcript might interfere with reverse transcription. The positions of the ends of the detected primer extension products (only for the products corresponding to the full length) are indicated by vertical arrows in Fig. 2. The downstream initiation site thus identified was one base upstream of the 5′-end of PLA_2 cDNA determined by the one-sided PCR method (20,25). To rule out the possibility of the longer primer extended products being generated by artifacts due to undesirable reactions such as snap-back synthesis, we tried to amplify the 5′ end of the longer cDNA selectively with primers 5 and 6 (see Table 1 and Fig. 2) by PCR using a cDNA template prepared with primer 1 as described in MATERIALS AND METHODS. PCR amplification with primers 5 and 6 gave an ethidium bromide-stained band of the expected size (about 200 bp) on an agarose gel (Fig. 6), indicating that the longer transcript does exist. The observed DNA was not derived from contaminating rat genomic DNA but from cDNA, since the PCR products derived from genomic DNA templates should be of larger size (about 800 bp) than the expected one due to the presence of an intron between the binding sites of primers 5 and 6 (Fig. 6).

As shown in Fig. 5, we analyzed various kinds of RNAs isolated from SMCs, ileum and spleen. Basically, all the samples gave the same pattern with different intensities, although the bands moved slightly faster than in other lanes when a large amount of SMCs RNA template (160 μg) was reverse transcribed, probably due to an electrophoretic artifact caused by overloading. The signal strength observed in Fig. 5 agreed with the result of RNA blotting analysis described above. The ratio of the signal intensity of the shorter and longer primer extended products seemed to be independent of the treatments of SMCs with various stimulants. Furthermore, the same primer extended products were detected when ileum and spleen poly $(A)^+$ RNA were used as

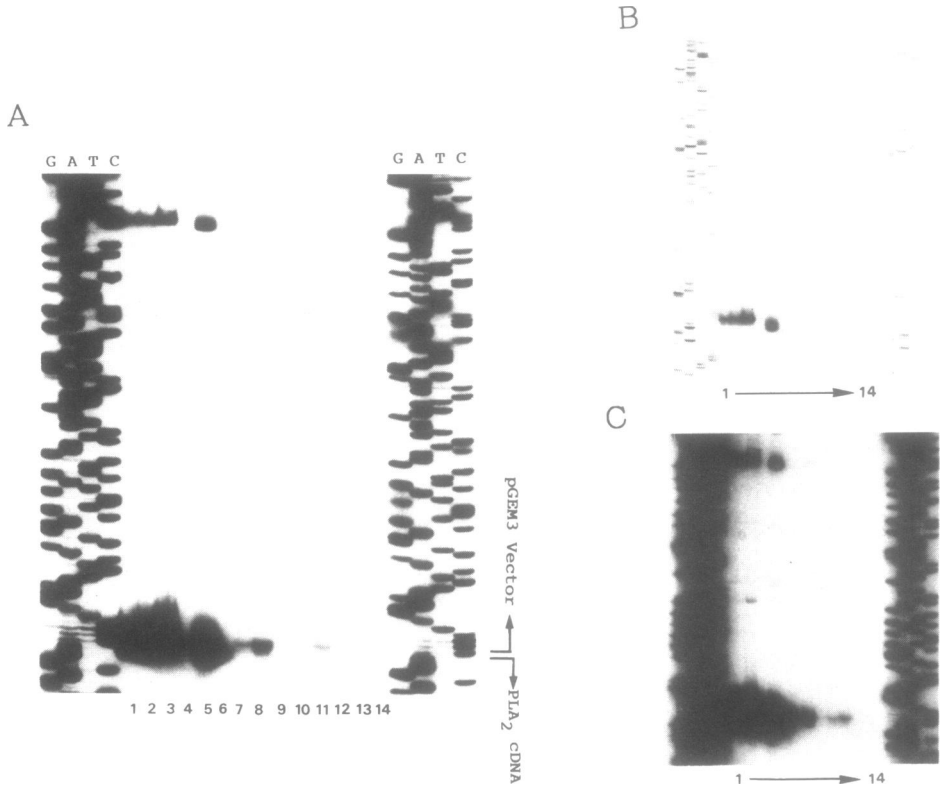

Figure 5. Detection of the transcription initiation sites of rat PLA_2 gene by hybridization-based primer extension method. After hybridization with ^{32}P-labeled oligonucleotide 2, the resultant membrane was exposed to X-ray film: A, two-day exposure of Kodak XAR film without intensifying screens; B, overnight exposure of Fuji RX film without intensifying screens; C, two-day exposure of Kodak XAR film with intensifying screens at −70°C. The sequencing ladders shown were derived from a cloned PLA_2 cDNA plasmid (PLA_2 cDNA was inserted into Sma I-Hind III site of pGEM3 vector) by dideoxy sequencing method using oligonucleotide 1 as a sequencing primer, and used as a size marker for assigning the size of the primer extended product. The sources and amounts of RNAs used for the primer extension reaction are as follows: lane 1, SMCs treated with LPS and forskolin, 20 μg; lane 2, SMCs treated with TNF and forskolin, 20 μg; lane 3, rat ileum poly $(A)^+$ RNA, 2 μg; lane 4, rat spleen poly $(A)^+$ RNA, 2 μg; lane 5, SMCs treated with forskolin, 160 μg; lane 6, vehicle SMCs, 160 μg; lane 7, SMCs treated with LPS and CHX, 20 μg; lane 8, SMCs treated with LPS, 20 μg; lane 9, SMCs treated with TNF and CHX, 20 μg; lane 10, SMCs treated with TNF, 20 μg; lane 11, SMCs treated with CHX and forskolin, 20 μg; lane 12, SMCs treated with forskolin, 20 μg; lane 13, SMCs treated with CHX, 20 μg; lane 14, vehicle SMCs, 20 μg. The concentrations of the stimulants are the same as those specified in the legend for Fig. 3.

templates as shown in Fig. 5. As reported previously, the ileum seemed to be a very rich source of PLA_2 (20).

The location of the major transcription start site thus identified was further verified by S1 nuclease mapping technique with end-labeled single-stranded probe, although the sensitivity was not high enough to detect the minor site (data not shown).

DISCUSSION

We have recently reported that PLA_2 mRNA levels are modulated by inflammation-related factors and cAMP in rat vascular SMCs (10), and further analyses have revealed that PLA_2 gene expression is mainly controlled at the transcriptional level (Ishizaki *et al.*, manuscript in preparation). We therefore considered it important to characterize the transcriptional unit of PLA_2 gene on the molecular basis. As the first step toward this end, we isolated the 5'-flanking region of this gene. The technique used to isolate the target region is a newly emerging one, adaptor ligation-mediated PCR, which saved much time and labor (13). The nucleotide sequence of the flanking region thus obtained is highly homologous to thıat of human PLA_2 gene around a putative TATA box, and almost identical to that reported by another group (26). We found that the isolated 5'-flanking region was capable of directing the expression of heterologous genes when they were placed downstream of it (data not shown).

While this study was in progress, the structure of rat PLA_2 gene was reported (26) with elucidation of the intron-exon structure of this gene, which is almost identical to that of human PLA_2 gene (8,9). However, only a short nucleotide sequence (314 bp) upstream of the 5'-most cDNA sequence was determined for the rat PLA_2 gene. Since our final goal is to understand the dynamics of the expression of PLA_2 gene in SMCs on the molecular basis, we need more detailed information about the transcriptional unit of the rat PLA_2 gene. In this respect, the next problem faced in the molecular characterization of the transcription unit is the precise mapping of the genomic sequence transcribed into RNA. Since the intron-exon structure of this gene has already been reported, the remaining problem is mapping the transcription initiation site of this gene, which has been only tentatively assigned for human and rat PLA_2 genes (8,9,26). In general, there are two widely used methods for determining transcription start sites; S1 nuclease mapping and primer extension (14). In this study, we selected an oligonucleotide-mediated primer extension technique because of its relative simplicity. However, in its conventional form, this method has relatively low sensitivity; since the amounts of the PLA_2 transcript in SMCs was not high enough to enable detection of the band on RNA blot with an end-labeled oligonucleotide probe unless SMCs were stimulated by a combination of factors, we anticipated difficulty in detecting the primer-extended products. Furthermore, a conventional primer extension technique with an end-labeled oligonucleotide primer requires careful preliminary examination of primer annealing conditions to remove spurious bands on detection. To overcome these two drawbacks, we developed a modified primer extension technique which takes advantage of the power of the genomic sequencing method. Although genomic sequencing was originally developed for *in vitro* footprinting and methylation analysis of eukaryotic genomic DNA, its high sensitivity without impairment of the resolution indicates its wide potential applicability. As demonstrated in this study, using the genomic sequencing method to detect of primer extended products made it possible to easily improve the sensitivity by preparing an oligonucleotide probe with high specific activity. Moreover, the detection specificity could be determined exclusively by the conditions of hybridization and subsequent washing, which allowed us to carry out the primer extension reaction even under relaxed primer annealing conditions (*e.g.*, use of a large molar excess of primer over template transcript, which shortens the annealing time required for completion of primer binding). With these improvements, we were able to detect the primer extended products from PLA_2 transcript without any difficulties. The sensitivity of our system should be at least ten times higher than that of a conventional primer extension technique with an end-labeled oligonucleotide primer, since the specific activity of a ^{32}P-dA tailed probe was more than ten times higher than that of an end-labeled one as demonstrated previously (22). Actually, we could visualize the primer extended products of RNA isolated from SMC treated with CHX or forskolin with only an overnight exposure using intensifying screens, indicating that the sensitivity of our primer extension analysis system is very close to that of RNA blotting analysis using a long cDNA probe labeled by random priming as shown in Fig. 3. Although our method still has the potential drawback of primer extension methods in general, *i.e.*, premature termination of reverse transcription, the method presented here can be recommended for various applications.

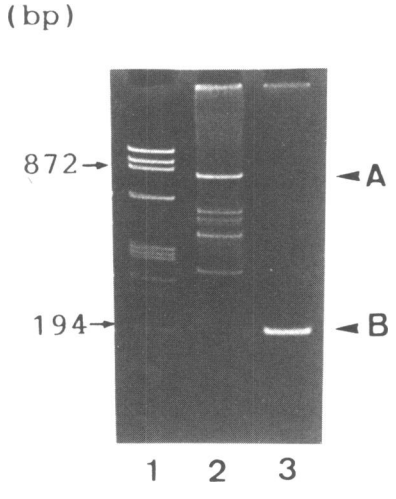

Figure 6. Confirmation of the presence of a longer PLA_2 transcript in SMCs. The number of PCR cycles were 30. PCR products (lanes 2 and 3) were analyzed on a 4% polyacrylamide gel. Arrows A and B indicate the bands with the expected sizes for the amplified products derived from genomic DNA and cDNA (about 800 bp and 200 bp, respectively). Lane 1, phiX DNA digested with Hae III; lane 2, genomic DNA template; lane 3, cDNA template generated with primer 1 by reverse transcription of total RNA isolated from SMCs treated with forskolin.

It is interesting to note that the 5'-most sequence of PLA_2 cDNA determined by the one-sided PCR method (20,25) was almost identical to the downstream major transcription initiation site assigned here. This suggests that the one-sided PCR method can be used as an alternative analytical method for mapping the transcription inititation site, although it might be difficult to detect minor transcription start sites as was the case with PLA_2 cDNA (20). If only a limited amount of RNA is available, this PCR-assisted method would be of great value.

RNA blotting analysis showed the consistency of our primer extension technique. Furthermore, the results revealed some important points. First, we observed that CHX was not an inhibitor but, in fact, an inducer of the elevation of PLA_2 mRNA levels in SMCs. Furthermore, when added in combination

with forskolin, CHX was found to synergistically potentiate cAMP-responsiveness. Although CHX has been described as stabilizing polysomal RNA nonspecifically, this side effect was relatively weak in our measurements of the amounts of total poly $(A)^+$ RNA in SMCs with and without CHX (data not shown). Moreover, CHX had no effects on intracellular cAMP concentrations at all. We hence concluded that the elevation of PLA_2 mRNA levels is attributable to the expected effect of CHX, *i.e.*, inhibition of protein synthesis. The potentiation of the effect of growth factors by CHX, an effect termed 'superinduction,' has been described for many genes (27–29). Subramaniam *et al.* reported that the c-*fos* serum-responsive element was sufficient to confer CHX-dependent inducibility upon a heterologous promoter, and suggested that serum-responsive enhancer elements were negatively regulated by one or more labile proteins (30). Although many cAMP-regulated genes have been isolated and characterized to date (31), little is known about 'superinduction' of these genes by CHX. Since the enhanced accumulation of PLA_2 mRNA by CHX suggests the existence of some short-lived negative regulators in SMCs, one possible explanation for this 'superinduction' of PLA_2 gene is to assume that these putative negative regulators can function in the pathway through which PLA_2 mRNA levels are controlled by cAMP. In contrast, we found the activation of PLA_2 gene by inflammation-related factors (TNF, IL-1, and LPS) was CHX-insensitive, although cAMP-responsiveness was potentiated by CHX as described above. The results, therefore, gave us another line of evidence that there are multiple distinct routes through which PLA_2 gene is activated, as proposed previously (10). Furthermore, we can exclude the possibility that LPS-induced PLA_2 gene activation is mediated through LPS-induced TNF and/or IL-1 gene activation (32,33), since the results imply that the activation of PLA_2 gene by inflammation-related factors as well as cAMP does not require preceding synthesis of other gene products. The elevation of PLA_2 mRNA levels is a direct primary response to these factors, although secondary response(s) could contribute to these phenomena to some extent.

Our results so far obtained show that PLA_2 gene is modulated by the following factors: cAMP, TNF, IL-1, LPS, and CHX (10). We also recently reported that glucocorticoids alleviate the cAMP-responsiveness of the PLA_2 gene (34). Furthermore, some cell-type specific regulators must be present since regulation patterns of this gene in rat pheochromocytoma cell line (PC-12), peritoneal macrophages and astrocyte cells are quite different from that in SMCs (Oka *et al.*, manuscript in preparation). These cell-type specific regulators would play a key role in tissue-specific expression of PLA_2 gene as reported previously (20). In order to further elucidate the regulation mechanism of PLA_2 gene expression, we are searching for *cis*-acting elements which can confer the responsiveness to these factors mentioned above. This course of study should throw light on understanding the dynamics of PLA_2 gene expression in the progression of vascular disorders such as inflammation.

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