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Cloning and Recombinant Expression of Phospholipase A_2 Present in Rheumatoid Arthritic Synovial Fluid*

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Synovial fluid from arthritic patients contains multiple forms of phospholipase A_2 (PLA_2), as resolved by high performance liquid chromatography (Seilhamer, J. J., Plant, S., Pruzanski, W., Schilling, J., Stefanski, E., Vadas, P., and Johnson, L. K. (1989) *J. Biochem. (Tokyo)*, submitted for publication). Here we describe the cloning of a human 4.5-kilobase gene and 800-base pair cDNA encoding the form representing the major peak of activity and protein mass (peak A). The clones encode a mature peptide of 124 amino acids, which follows a prepeptide of 20 residues. The deduced amino acid sequence constitutes an enzyme of the "Type II" class of PLA_2s, and resembles PLA_2s from other mammalian sources. This represents the first report of a full length mammalian non-pancreatic PLA_2 sequence. Active transcription of this PLA_2 gene was detected in two different inflammatory cell sources. Recombinant human peak A PLA_2 was expressed in vaccinia as a secreted protein which accumulated in conditioned medium.

Phospholipase A_2 (PLA_2)[1] plays a central role in liberating lysophosphatides and free fatty acids from membrane phospholipids, thereby initiating the production of eicosanoid mediators which profoundly influence inflammatory reactions. Evidence to date has shown that both membrane-associated and secreted (soluble) forms of PLA_2 are present in and produced by cells participating in the inflammatory reaction. These same enzymes, when sequestered in peritoneal or joint cavities, may accumulate to very high levels in diseases such as arthritis (1–6) and may play a role in such chronic inflammatory conditions. Consistent with this hypothesis are findings that injection in knee joints of purified synovial fluid PLA_2 at concentrations found *in vivo* caused acute inflammatory and subacute proliferative changes in synovial structures (5). Inhibition of the specific PLA_2 enzyme(s) present in arthritic synovial fluid could represent a possible point of therapeutic intervention in such inflammatory disorders.

Despite the extensive knowledge of structure and enzymatic mechanism of the relatively abundant venom and mammalian pancreatic PLA_2s (7–10), relatively little is known about other mammalian PLA_2 enzymes. The amino-terminal portion of the amino acid sequences of several mammalian non-pancreatic PLA_2s have been determined, including enzymes isolated from porcine intestine (11), rabbit ascites (12), rat platelet (13), rat spleen (14), and rat peritoneal exudate (15). Recently, amino-terminal sequences for human synovial fluid PLA_2 (16)[2,3] and another putative PLA_2 sequence of unknown function (19) have become the first human non-pancreatic PLA_2 sequences available. This information has yielded much insight into relative form, function, and identity of these mammalian PLA_2 isolates. On the other hand, information about the other two-thirds of these molecules, which contains both the active site regions and many important structural and functional determinants has been nonexistent. Furthermore, the extrapolation of the biochemical properties and sequences of other mammalian enzymes to a form relevant to human disease has been, at best, ambiguous. An additional limitation is that without PLA_2 form-specific probes, it has been difficult to attribute biological effects to specific enzyme forms.

The isolation and characterization of PLA_2 from human arthritic synovial fluid has been detailed previously (20–21). Upon further purification of the enzyme on high performance liquid chromatography, we were able to resolve the enzyme into multiple peaks of activity, each with distinct biochemical properties.[2] The fraction eluting earliest, peak A, constituted the majority of activity and protein mass present in the extract. A second most abundant fraction (peak B), could readily be distinguished from the former by its enhanced activity in 0.5 M Tris and/or 0.2% sodium deoxycholate. In the present work we describe the isolation of both genomic and cDNA clones encoding the peak A PLA_2 sequence. Also, we have examined transcription of this gene in cells obtained from inflamed sources. Finally, we have demonstrated recombinant expression from the peak A cDNA of a secreted active PLA_2 with properties similar to the native enzyme.

EXPERIMENTAL PROCEDURES

Library Screening—10^6 clones from a human genomic library (Clonetech, Inc.) were lifted onto nitrocellulose filters, denatured, baked at 80 °C 2 h, prehybridized for 4 h in 20% formamide, 6 × SSC (1.0 M NaCl, 0.1 M sodium citrate), 1 × Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), and 0.1% sodium dodecyl sulfate (SDS) at 37 °C, and hybridized overnight in the same solution with 10^6 cpm/filter of ^{32}P-labeled oligonucleotide probes, with each probe on one of two duplicate lifts. The filters were washed in 1 × SSC, 0.1% SDS at 60 °C for 60 min and autoradiographed overnight.

RNA Blot Hybridization—Total cell RNAs were isolated using the method of Gubler and Hoffman (22) and were electrophoresed on a

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J04704.

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[1] The abbreviations used are: PLA_2, phospholipase A_2; SDS, sodium dodecyl sulfate.

[2] J. J. Seilhamer, S. Plant, W. Pruzanski, J. Schilling, E. Stefanski, P. Vadas, and L. K. Johnson, *J. Biochem. (Tokyo)*, submitted for publication.

[3] Kramer, R., Hession, C., Johansen, B., Hayes, G., McGray, P., Chow, E. P., Tizard, R., and Pepinsky, R. B. (1989) *J. Biol. Chem.*, in press.

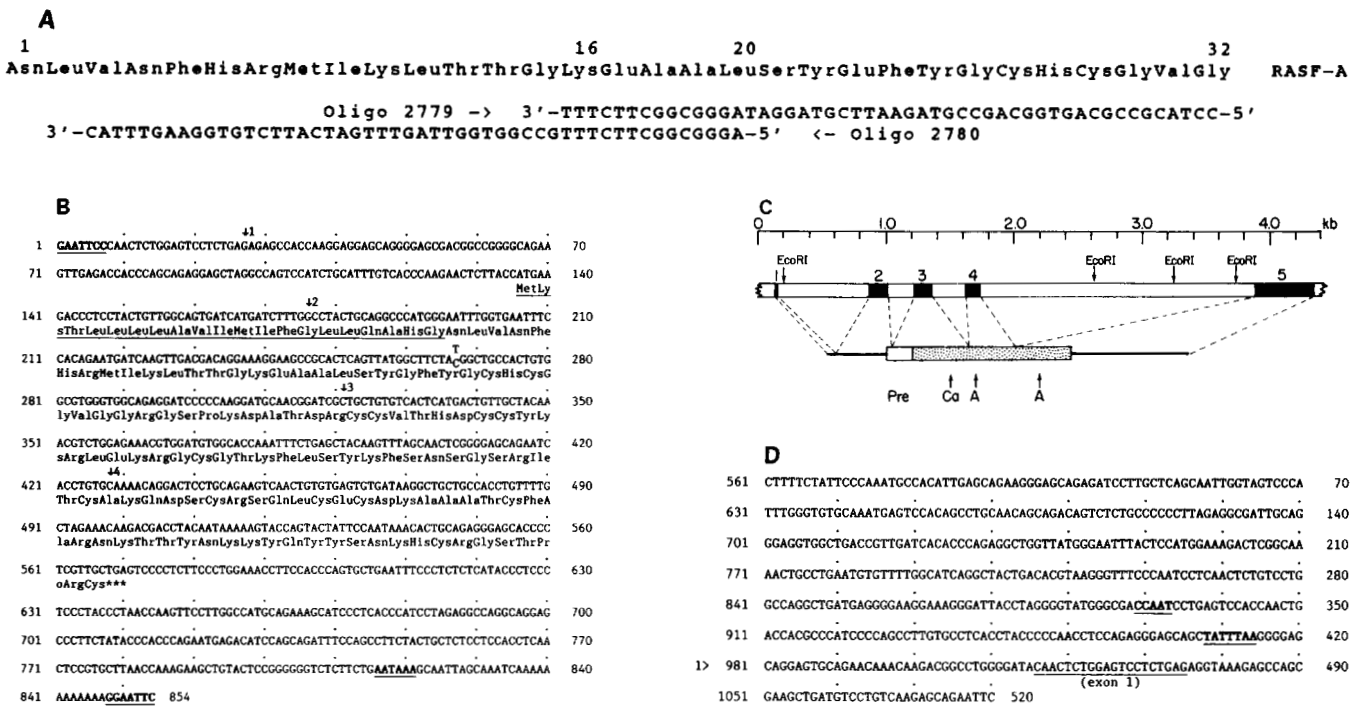

FIG. 1. **Complementary strand screening probes (*A*); peak A cDNA sequence (*B*); gene and cDNA structure (*C*); exon 1 sequence (*D*).** *A*, 45-mer oligonucleotide probes were designed based upon amino acid sequence determined in Footnote 2. RASF-A, rheumatoid arthritic synovial fluid (peak A). *B*, shown is the sequence of cDNA clone 4, which encodes peak A PLA_2 (translated region). The positions of the 4 introns are indicated (↓), and they comprised 700 (*1*), 208 (*2*), 257 (*3*), and 2061 (*4*) bases. Base 277, apparently a polymorphic base, was a "C" in clone 4 and a "T" in other cDNA clones. The poly(A) addition site, *Eco*RI linkers, and prepeptide are *underlined*. *C*, the five regions of the gene matching cDNA clone 4 are represented in diagramatic form. Nontranslated (*line*), translated (*boxed*), and prepeptide (*Pre*) regions of the cDNA are represented, along with the calcium-binding (*Ca*) and active site (*A*) regions of the enzyme. *D*, DNA sequence of the region of the gene containing exon 1 and flanking sequence. Putative transcriptional control elements, TATTTAA (408–414) and CCAAT (329–333), are highlighted. *kb*, kilobases.

1.0% agarose, 18% formaldehyde gel, transfer blotted to a nitrocellulose filter, baked at 80 °C 2 h, and processed as above with 10^7 cpm of ^{32}P-labeled oligonucleotide probe. The filter was washed in 1 × SSC, 0.1% SDS at 60 °C for 60 min and autoradiographed for 3 days.

cDNA Library Production—Human peritoneal exudate cDNA was synthesized using a kit (Boehringer Mannheim) and was cloned into λgt10 vector arms (Stratagene Inc.) via *Eco*RI linkers. The resulting clones were screened as described above.

PLA_2 Activity Assay—PLA_2 activity was measured using a phosphatidylcholine/sodium deoxycholate mixed micele assay system as described.[2]

RESULTS AND DISCUSSION

Using the 25-residue amino-terminal amino acid sequence we obtained from material purified from human synovial fluid,[2] recently confirmed elsewhere (16), and by making allowances for conserved Cys and Gly residues, two partially overlapping 45-mer oligonucleotide probes were designed (Fig. 1*A*). Since the cellular source of synovial fluid PLA_2 was unknown, a genomic library was screened first to obtain the nucleotide sequence, and then non-degenerate probes made specific for the sequence would subsequently be used to find an mRNA source. A human genomic library was screened for coincident hybridization signals to both probes. Six such signals were detected, and the clones were purified through additional rounds of screening. Restriction digest analysis of the cloned DNAs revealed that they were identical. An *Alu*I fragment containing the hybridizing DNA (Fig. 1*B*, bases 202–287) was subcloned into M13 and the DNA sequence was determined. The DNA sequence of the *Alu*I fragment was found to contain a region encoding the correct amino acid sequence. It also contained the remainder of the coding exon (bases 176–320), including a sequence resembling the Ca^{2++}-binding loop (bases 265–282), conserved in known PLA_2s (7, 8). The presence of the remainder of the exon confirmed that the clone contained a PLA_2 gene.

In order to screen various sources for the presence of transcripts from this gene, a 60-base oligonucleotide matching the DNA sequence of the genomic clone was synthesized. RNAs from various sources, including phorbol ester-induced human cell lines U937 and HL60, inflamed human synovial tissue, and a cell pellet from a human peritoneal exudate (containing mononuclear and polymorphonuclear cells), along with RNA from several other mammalian tissues and cell lines were prepared and blotted onto nitrocellulose filters. As seen in Fig. 2, the presence of an ~800-base transcript was detected only in the RNA from synovial tissue and the peritoneal exudate cells. In the exudate cell RNA the signal was observed in much greater relative abundance. From this, it would appear that the gene encoding this PLA_2 form is actively transcribed in cells associated with two different inflammatory disorders. Interestingly, no signal was detected in RNA from the two induced human promyelocytic cell lines, U937 and HL60, suggesting these cells could contain a different PLA_2.

The relative abundance of signal in the RNA from peritoneal exudate cells suggested it would make the best cDNA source. A cDNA library was prepared and then screened using the 60-base oligonucleotide probe described in Fig 2. Sixteen signals were obtained from 2 × 10^5 plaques, and 4 of the clones were purified further. The DNA sequence of the longest of these clones (number 4) is represented in Fig. 1*B*, along

FIG. 2. **Northern blot of RNA from various sources.** Electrophoresed RNA was prepared from U937 (*lane 4*) and HL60 cells (*lane 3*) induced with 0.16 μM 12-*O*-tetradecanoylphorbol 13-acetate, HL60 cells induced with 0.1 nM 1,25-dihydroxyvitamin D_3 (*lane 1*) and 1.25% dimethyl sulfoxide (*lane 2*), inflamed human synovial tissue (*lane 6*), a cell pellet from a peritoneal exudate from a nonsterile peritonitis patient (*lane 7*), porcine jejunum (*lane 9*), pancreas (*lane 12*), and spleen (*lane 11*), and rat liver (*lane 10*). The blot was probed with a 60-base oligonucleotide probe represented by the complementary strand of bases 208–267 in Fig. 1*B*. *Lanes 5* and *8* contained molecular weight standards.

with the encoded sequence of the enzyme. The coding regions of the genomic clone were determined by matching the sequence with the cDNA (see Fig. 1*C*). The gene spans 4.6 kilobases and contains 5 exons. Introns 2, 3, and 4 fall in precisely the same positions within the sequence as they occur in the gene encoding pancreatic PLA_2 (23). The first intron occurred 107 bases upstream from the initiating Met, and interrupts the 5′-noncoding region of the cDNA. In the gene sequence (Fig. 1*D*), a TATA-like sequence (TATTTAA) was found 41 bases upstream from the start of the cDNA sequence. Another putative transcriptional control signal, CCAAT, was located 121 bases upstream. The precise localization of the transcriptional start site will require primer extension analysis; however, the presence of these putative transcriptional signals suggests that it is unlikely to be significantly upstream from the start of the cDNA clone.

The cDNA clone 4 encoded a mature protein with a calculated molecular mass of 13,939 daltons, taking into account the amino terminus observed from the protein sequence. The amino acid sequence was aligned in Fig. 3 with other relevant published PLA_2 sequences. Its sequence bears a striking resemblance to those of other known PLA_2s. First, like the other non-pancreatic mammalian enzymes, the placement of its Cys residues follows a Type II pattern, consistent with its proinflammatory nature. Notably, the key residues implicated in the hydrolytic mechanism of other PLA_2 enzymes (8, 9), including Phe[5], Ile[9], His[48], Asp[49], Asp[99], Ala[102], and Ala[103] are present in the clone 4 sequence. The Ca^{2++}-binding loop (residues Tyr[28]-Gly[32]) is entirely conserved except for His[31], the only variable position within the loop. Overall, its spacing and Cys residues exactly match the sequence from *Crotalus* venom, except for the short α-helical region immediately following the active site (residues 52–56). In this region, the spacing of residues more closely resembles the pancreatic enzyme. This sequence contains, like other PLA_2s with strong anticoagulant activity (27), a strong net positive charge within

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Exon 2:                  1        10        20        30        40
p Ileum (11)             DLLNFRKMIK-LKTGKAPVPNYAFYGCYCGLGGKGSPKDATD?
rab Ascites (12)         HLLDFRKMIR-YTTGKEAT?SYGAYGCSCGVGGR?APK?A
rat (13-15)              ?LLEFGQMIL-FKTGKRADVSYGFYGCHCGVGGRGSP
h RASF-A                 NLVNFHRMIK-LTTGKEAALSYGFYGCHCGVGGRGSPKDATDR
h NP (19)                SFWQFQRRVK-HITGRSAFFSYGYGCYCGLGDKGIPVDDTDR
h Panc (23,24)           AVWQFRKMIKCVIPGSDPFLEYNNYGCYCGLGGSGTPVDELDK
C. atrox (25)            SLVQFETLIM-KIAGRSGLLWYSAYGCYCGWGGHGLPQDATDR
A. pisc "K-49" (26)      SVLELGKMIL-QETGKNAITSYGSYGCNCGWGHRGQPKDATDR

Exon 3:                  44    50        60        70        80   85
H RASF-A                  CCVTHDCCYKRLEKR-GC-----GTKFLSYKFSNSGSRITC-
h panc                    CCQTHDNCYDQAKKLDSCKFLLDNPYTHTYSYSCSGSAITCS
C. atrox                  CCFVHDCCYG---KATDC-----NPKTVSYTYSEENGEIIC-
A. pisc                   CCFVHKCCYK---KLTDC-----NHKTDRYSYSWKNKAIIC-

Exon 4:          86  90        100       110       120       130
rab ascites                                        QFYPANRCSGRPPSC
h RASF-A          AKQDSCRSQLCECDKAAATCFARNKTTYNKKYQYYSNKHCRGSTPRC
h panc            SKNKECEAFICNCDRNAAICFSKAPYNKAHK-NLDTKKYCQS
C. atrox          GGDDPCGTQICECDKAAAICFRDNIPSYDNKYWLFPPKDCREEPEPC
A. pisc           EEKNPCLKEMCECDKAVAICLRENLDTYNKKYKAYFKLKCK-KPDTC
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FIG. 3. **Homology of known PLA_2 sequences.** The amino acid sequence deduced from peak A cDNA clone 4 is shown, aligned by exons, with other known PLA_2 sequences from porcine ileum (*p Ileum;* 11), rabbit ascites (*rab Ascites;* 12), rat platelet (13), spleen (14), and peritoneal exudate (15; *rat*), "non-pancreatic" PLA_2 (*NP;* 19), human pancreas (*h panc;* 23, 24), and *Crotalus atrox* (*C. atrox;* 25) and *Agkistrodon piscivorus* "K-49" venom (*A. pisc;* 26). *h RASF-A,* human rheumatoid arthritic synovial fluid, peak A.

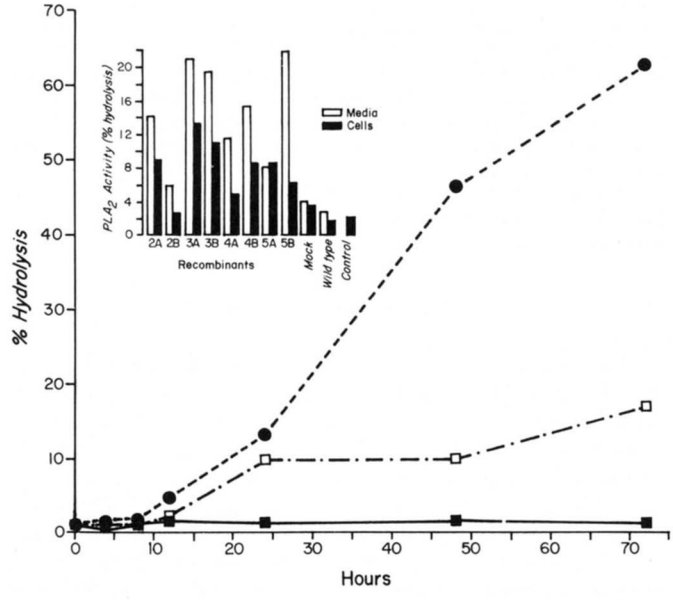

FIG. 4. **Recombinant expression of peak A PLA_2.** Serum-free medium conditioned during a 4-h collection, harvested 48 h after viral infection, and lysed cells were assayed from each of four recombinant vaccinia clones (*inset*). PLA_2 levels present in conditioned media sampled at indicated times following infection of CV-1 cells by wild type vaccinia (*solid squares*) and recombinant vaccinia/PLA_2 clone 4B were assayed under standard (*solid circle*) and 0.5 mM Tris (*open square*) conditions.[2] Activity is expressed as percent hydrolysis of ^{14}C-labeled 2-arachidonyl phosphatidylcholine per 10 μl of media under standard assay conditions.[2]

this region. Within the amino-terminal 15 residues, 4 positively charged residues (His^6, Arg^7, Lys^{10}, and Lys^{15}) occur. If this region folds into an α-helix as proposed for other PLA_2s (7–9, 12), these 4 positively charged residues would align along one side of the helix. Positively charged residues occurring in this manner have been proposed as determinants of responsiveness to the "bactericidal/permeability-increasing protein isolated by Elsbach *et al.* (12, 28). Upstream from the 124-residue mature coding sequence lies a 20-residue peptide which most likely represents a signal sequence for membrane translocation (29). A proenzyme segment was not evident within this region, suggesting the enzyme is probably not secreted as a zymogen (trypsin-activated) as is the case for pancreatic PLA_2 (30).

Recombinant expression of the cloned gene fragments in the vaccinia virus system (17) represented an appropriate method to test whether this gene encoded an active secreted PLA_2. The cDNA clone 4 was trimmed of its 5′- and 3′-noncoding DNA through digestion at *Sac*I and *Hin*dIII sites (bases 125–130 and 588–593, respectively), both of which were engineered into the sequence via oligonucleotide-directed mutagenesis. The resulting coding segment was blunted by fill-in synthesis and cloned into the *Sma*I site of plasmid pSC11 (18). Purified plasmid DNA was co-transfected into CV-1 cells with wild type vaccinia viral DNA, and the resulting recombinants were screened by selection in medium containing 5′-bromodeoxyuridine. Four recombinant vaccinia clones were selected for analysis by quantitating secreted and cell-associated PLA_2 activity (Fig. 4, *inset*). Clone 5B, showing the highest media PLA_2 levels was chosen for further study. In a subsequent larger culture, media samples taken at various times after viral infection were assayed for PLA_2 activity. As seen in Fig. 4, PLA_2 activity from the PLA_2/vaccinia recombinant virus was primarily extracellular and accumulated linearly in the medium over 72 h, after which viral cell lysis became significant. The expressed PLA_2 activity retained the property of sensitivity to Tris inhibition exhibited by the native enzyme.[2]

In summary, a cloned cDNA encoding the major PLA_2 species present within rheumatoid synovial fluid has been obtained, and when expressed in the vaccinia expression system yields a secreted enzyme retaining properties of the native enzyme. While this form may not be the only PLA_2 present in all forms of arthritis, it clearly represents the most prevalent and active form found in the types examined. Its abundance in peritoneal exudate cells suggests this enzyme occurs systemically and could play a role in many types of acute inflammatory disorders. Further work will be necessary to determine the cell type(s) from which this enzyme originates, and to elucidate its precise role in the perpetuation of inflammatory disorders.

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Note Added in Proof—Since submission of this manuscript, Lai and Wada (Lai, C., and Wada, K. (1988) *Biochem. Biophys. Res. Commun.* **157,** 488–493) have reported a PLA_2 isolate from synovial fluid with identical sequence. Also, Hayakawa *et al.* (Hayakawa, M., Kudo, I., Tomita, M., Nojima, S., and Inoue, K. (1988) *J. Biochem. (Tokyo)* **104,** 767–772) have reported a complete amino acid sequence for a PLA_2 from rat platelets.

REFERENCES

1. Pruzanski, W., Vadas, P., Stefanski, E., and Urowitz, M. B. (1985) *J. Rheumatol.* **12,** 211–216
2. Vadas, P., Wasi, S., Movat, H. Z., and Hay, J. B. (1981) *Nature* **293,** 583–585
3. Pruzanski, W., Vadas, P., Kim, J., Jacobs, H., and Stefanski, E. (1989) *J. Rheumatol.* **15,** 791–794
4. Vadas, P., and Pruzanski, W. (1984) *Adv. in Inflam. Res.* **7,** 51–59
5. Vadas, P., Pruzanski, W., Kim, J., and Fornasier, V. (1989) *Am. J. Pathol.*, in press
6. Vishwanath, B. S., Fawzy, A. A., and Franson, R. C. (1988) *Inflammation* **12,** 549–561
7. Dufton, M. J., Eaker, D., and Hider, R. C. (1983) *Eur. J. Biochem.* **137,** 537–544
8. Dufton, M. J., and Hider, R. C. (1983) *Eur. J. Biochem.* **137,** 545–551
9. Renetseder, R., Brunie, S., Dijkstra, B. W., Drenth, J., and Sigler, P. B. (1985) *J. Biol. Chem.* **260,** 11627–11634
10. Slotbloom, A. J., Verheij, H. M., and De Haas, G. H. (1982) *New Comp. Biochem.* **4,** 354–434
11. Verger, R., Ferrato, F., Mansbach, C. M., and Pieroni, G. (1982) *Biochemistry* **21,** 6883–6889
12. Forst, S., Weiss, J., Elsbach, P., Maranganore, J. M., Reardon, I., and Heinrikson, R. L. (1986) *Biochemistry* **25,** 8381–8385
13. Chang, H. W., Kudo, I., Tomita, M., and Inoue, K. (1987) *J. Biochem. (Tokyo)* **102,** 147–154
14. Ono, T., Tojo, H., Kuramitsu, S., Kagamiyama, H., and Okamoto, M. (1988) *J. Biol. Chem.* **263,** 5732–5738
15. Hayakawa, M., Horigome, K., Kudo, I., Tomita, M., Nojima, S., and Inoue, K. (1987) *J. Biochem. (Tokyo)* **101,** 1311–1314
16. Hara, S., Kudo, I., Matsuta, K., Miyamoto, T., and Inoue, K. (1988) *J. Biochem. (Tokyo)* **104,** 326–328
17. Mackett, M., and Smith, G. L. (1986) *J. Gen. Virol.* **67,** 2067–2082
18. Chakrabarti, S., Brechling, K., and Moss, B. (1985) *Mol. Cell Biol.* **5,** 3403–3409
19. Seilhamer, J. J., Randall, T. L., Johnson, L. K., Heinzmann, C., Klisak, I., Sparkes, R. S., and Lusis, A. J. (1989) *J. Cell. Biochem.* **39,** 327–337
20. Stefanski, E., Pruzanski, W., Sternby, B., and Vadas, P. (1986) *J. Biochem. (Tokyo)* **100,** 1297–1303
21. Vadas, P., Stefanski, E., and Pruzanski, W. (1985) *Life Sci.* **36,** 579–587
22. Gubler, U., and Hoffman, B. J. (1983) *Gene (Amst.)* **25,** 263–269
23. Seilhamer, J. J., Randall, T. L., Yamanaka, M., and Johnson, L. K. (1986) *DNA (N. Y.)* **5,** 519–527
24. Verheij, H. M., Westerman, J., Sternby, B., and De Haas, G. (1983) *Biochim. Biophys. Acta* **747,** 93–99
25. Verheij, H. M., Egmond, M. R., and de Haas, G. H. (1981) *Biochemistry* **20,** 94–99
26. Maranganore, J., and Heinrikson, R. L. (1986) *J. Biol. Chem.* **261,** 4797–4804
27. Kini, R. M., and Evans, H. J. (1987) *J. Biol. Chem.* **262,** 14402–14407
28. Elsbach, P., Weiss, J., Franson, R., Beckerdite-Quagliata, S., Schneider, A., and Harris, L. (1979) *J. Biol. Chem.* **254,** 11000–11009
29. Von Heijne, G. (1983) *Eur. J. Biochem.* **133,** 17–21
30. Grataroli, R., Dijkman, R., Ditulh, C. E., Van Der Ouderaa, F., De Haas, G. H., and Figarella, C. (1982) *Eur. J. Biochem.* **122,** 111–117