and the selectable marker *Ecogpt*. The metastatic potential of these lines requires further examination in the presence of E1a expression.

Another explanation for the difference in metastatic potential observed between one- and two-gene transformants is that different populations of rat embryo cells are transformed by the *ras* gene alone, unlike the situation when *ras* is cotransfected with E1a. It is possible that only a small subset of the heterogeneous mixture of rat embryo cells is susceptible to transformation by the *ras* oncogene alone. This subset of cells may also be predisposed toward the metastatic phenotype. Single gene–transformed cell lines show normal diploid karyotypes (*20*), suggesting that gross chromosomal abnormalities or rearrangements are not a feature of cell types that are susceptible to transformation by the *ras* oncogene alone. When two cooperating oncogenes are cotransfected, the increase in the frequency of morphological transformation could be the result of transformation of a broader spectrum of embryo cell types. Cell types predisposed to metastasis should be present in the population of cells transformed by *ras* and E1a, but the percentage of such cells would be low (*21*).

NIH3T3 cells transformed by the *ras* oncogene are metastatic in nude mice (*22*). We have demonstrated that the *ras* oncogene can transform early passage rat embryo cells into tumorigenic cells that are highly metastatic in an immunodeficient animal. Thus, normal cells with a limited potential for growth in culture have become established, have been transformed, and have acquired metastatic potential apparently through the action of a single oncogene. Although the *ras* oncogene can induce morphological transformation, we do not yet know whether *ras* confers or induces the properties of establishment and metastasis or whether they are present in the recipient cell.

REFERENCES AND NOTES

1. C. Shih, B. Shilo, M. Goldfarb, A. Dannenberg, R. Weinberg, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5714 (1979).
2. T. Krontiris and G. Cooper, *ibid.* **78**, 1181 (1981).
3. H. E. Varmus, *Annu. Rev. Genet.* **18**, 553 (1984).
4. A. Houweling, P. J. Van den Elsen, A. J. van der Eb, *Virology* **105**, 537 (1980).
5. R. Bernards, A. Houweling, P. I. Schier, J. Bos, A. J. van der Eb, *ibid.* **120**, 422 (1982).
6. M. Rassoulzadegan *et al.*, *Nature (London)* **300**, 713 (1982).
7. M. Rassoulzadegan *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4354 (1983).
8. H. E. Ruley, *Nature (London)* **304**, 602 (1983).
9. H. Land, L. Parada, R. Weinberg, *ibid.*, p. 596.
10. D. Spandidos and N. Wilkie, *ibid.* **310**, 469 (1984).
11. G. H. Yoakum *et al.*, *Science* **227**, 1174 (1985).
12. L. Parada, C. Tabin, C. Shih, R. Weinberg, *Nature (London)* **297**, 474 (1982).
13. D. Capon, E. Chen, A. Levinson, P. Seeburg, D. Goeddel, *ibid.* **302**, 33 (1983).
14. C. Gorman, R. Padmanabhan, B. H. Howard, *Science* **221**, 551 (1983).
15. M. Imperiale, L. Feldman, J. Nevins, *Cell* **35**, 127 (1983).
16. C. Der and G. Cooper, *ibid.* **32**, 201 (1983).
17. J. L. Cook and A. M. Lewis, Jr., *Science* **224**, 612 (1984).
18. J. L. Cook, J. B. Hibbs, A. M. Lewis, *Int. J. Cancer* **30**, 795 (1982).
19. Y. Sawada, B. Föhring, T. Shenk, K. Raška, *Virology* **147**, 413 (1985).
20. R. J. Muschel, K. Nakahara, E. Chu, R. Pozzatti, L. J. Liotta, in preparation.
21. We estimate that only 2 percent of the population of two-gene transformants would exhibit high metastatic potential based on the tenfold difference in frequency of transformation and the fivefold difference in efficiency of establishment in culture observed between cells transformed by *ras* versus *ras* plus E1a.
22. U. P. Thorgeirsson *et al.*, *Mol. Cell. Biol.* **5**, 259 (1985); S. C. Bernstein and R. A. Weinberg, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1726 (1985); R. G. Greig *et al.*, *ibid.*, p. 3698.
23. F. Graham and A. van der Eb, *Virology* **52**, 456 (1973); L. Laimins, P. Gruss, R. Pozzatti, G. Khoury, *J. Virol.* **49**, 183 (1984).
24. P. J. Southern and P. Berg, *J. Mol. Appl. Genet.* **1**, 327 (1982).
25. D. DiMaio and D. Nathans, *J. Mol. Biol.* **140**, 129 (1980).

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Diverse Gene Expression for Isotypes of Murine Serum Amyloid A Protein During Acute Phase Reaction

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Serum amyloid A protein (SAA) is a precursor for a major component of amyloid fibrils, which, upon deposition, cause secondary amyloidosis in diseases such as rheumatoid arthritis. In mice, SAA is encoded by at least three genes, which show diverse expression during inflammation. Furthermore, in amyloidosis-resistant SJL mice, the gene expression for one SAA isotype, SAA2, is defective, although SAA2 gene expression is normal in amyloidosis-susceptible BALB/c mice. Because only SAA2-derived products deposit in mouse amyloid tissues, the resistance of SJL mice to amyloidosis seems to be due to defective SAA2 gene expression. Thus, the study emphasizes the importance of SAA gene structure in determining susceptibility to amyloidosis.

SECONDARY AMYLOIDOSIS, A SERIOUS complication of chronic inflammatory diseases such as rheumatoid arthritis, juvenile arthritis, and ankylosing spondylitis, is caused by the formation in tissues of amyloid fibrils. The major component of amyloid fibrils is derived from serum amyloid A protein (SAA). SAA is the most prominent acute phase reactant, and high SAA concentrations are frequently found in various inflammatory diseases, thus predisposing the patient to amyloid deposition (*1*). However, the normal physiological function of SAA is not known, although SAA may bind to a lipopolysaccharide portion of bacterial cell walls and may function in clearance of bacteria (*2*). We have recently isolated and characterized complementary DNA (cDNA) clones encoding two major SAA isotypes of BALB/c mice—SAA1 [11.75 kilodalton (kD)] and SAA2 (11.65 kD)—which show a 95 percent homology in their cDNA sequences (*3*). We report here that the mouse genome contains at least three SAA genes and that, while the SAA1 and SAA2 genes are expressed at high levels during acute phase reactions, the gene for the third SAA isotype (SAA3) is expressed only at a very low level. We further show that the SAA2 gene expression is meager in SJL mice. Since only the SAA2-derived product is deposited in mouse amyloid tissues (*4*), the resistance of SJL mice to azocasein-induced amyloidosis is probably due to defective SAA2 gene expression.

Mouse SAA2 cDNA was used to screen the BALB/c mouse Hae III genomic library in phage Charon 4A (*5*), and four positive phage clones were isolated. On the basis of their hybridization with the SAA2- and SAA1-specific synthetic oligonucleotide probes (Fig. 1A), the two clones λ5-12 and λ39-2 were identified as carrying the SAA2 and SAA1 genes, respectively (Fig. 2). In addition, while the λ5-12 clone contained a Xho I site 250 base pairs (bp) downstream of the Eco RI site within the SAA2 coding region, the λ39-2 clone contained a Stu I site near the Eco RI site in the SAA1 coding region: these restriction sites are unique to the SAA2 and SAA1 cDNA's, respectively (*3*). To define a possible linkage of the

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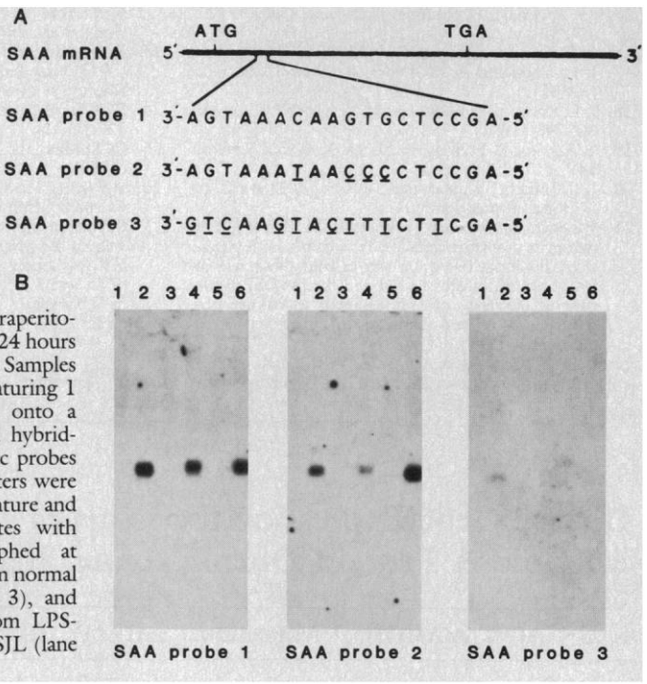

Fig. 1. (A) Base sequences of synthetic oligonucleotide probes. SAA probes 1, 2, and 3 are complementary to SAA1, SAA2, and SAA3 mRNA (*3*). Base differences are underlined. (B) Northern blot analysis with synthetic oligonucleotide probes specific for SAA1, SAA2, and SAA3 mRNA. Polyadenylated RNA was prepared from liver of normal mice or mice given an intraperitoneal injection of LPS (50 μg) 24 hours before RNA preparation (*15*). Samples (7.5 μg) were sized on a denaturing 1 percent agarose gel, blotted onto a nitrocellulose filter (*16*), and hybridized with radioactive synthetic probes at 50°C for 16 hours. The filters were washed twice at room temperature and once at 50°C for 5 minutes with 6× SSC, and autoradiographed at −70°C. RNA samples are from normal BALB/c (lane 1), SJL (lane 3), and Swiss (lane 5) mice and from LPS-stimulated BALB/c (lane 2), SJL (lane 4), and Swiss (lane 6) mice.

SAA1 and SAA2 genes, a 300-bp probe (probe C in Fig. 2) was prepared from the 5′ end of the λ5-12 clone and was used in hybridization with the λ39-2 clone. The 5′ end of the λ5-12 clone hybridized to the 3′ end of the λ39-2 clone, thus establishing that the SAA1 and SAA2 genes are directly adjacent in divergent transcriptional orientation (Fig. 1). The other two overlapping clones, λ1-7 and λ42-12, hybridized with an SAA2 cDNA probe only under conditions of low stringency, but were effectively hybridized with the SAA3 specific synthetic oligonucleotide probe (Fig. 1A). The SAA3 cDNA clone was originally isolated by Morrow *et al.* (*6*) and has a 70 to 72 percent nucleotide sequence homology with SAA1 and SAA2 cDNA in the coding regions (*3, 7*). The corresponding genomic clone was also isolated and characterized: in Southern blot analysis with an SAA3 cDNA probe the clone gave single hybridizing fragments upon being digested with Eco RI (7.1 kb), Hind III (8.2 kb), Bam HI (2.9 kb), Xba I (1.1 kb), and Bgl II (4.4 kb), respectively (*7*). Comparison of these data with the restriction map (Fig. 2) constructed from the inserts of the λ1-7 and and λ42-12 clones confirms that these two clones contain the SAA3 gene.

To study further the organization of the SAA gene in the mouse chromosomal DNA, we carried out Southern blot analysis of liver DNA from BALB/c mice with a 3.4-kb Xba I fragment from the SAA2 genomic clone (probe A in Fig. 2) and a 1.1-kb Xba I fragment from the SAA3 genomic clone (probe B in Fig. 2) as hybridization probes. As shown in Fig. 3, lane A, probe A hybridized to two genomic fragments in Southern blots made with four different restriction enzymes (Eco RI, Bam HI, Bgl II, Sac I): two bands were also observed in Southern blots of Bcl I and Kpn I digested DNA. In each case, one of two fragments hybridized with probe A (3-kb Eco RI, 6.1-kb Bam-HI, 3.9-kb Bgl II, and 7.8-kb Sac I fragments) had the fragment length characteristic of the SAA2 gene; the other fragments (6.2-kb Eco RI, 10.5-kb Bam HI, 8.8-kb Bgl II, and 4.2-kb Sac I fragments) were thought to be derived from the SAA1 gene (Fig. 2) (Bgl II sites are not shown). The hybridization signals were removed by boiling the nitrocellulose filter for 5 minutes in 10 m*M* tris–1 m*M* EDTA (*p*H 8.0), and the filter was rehybridized with probe B. The resulting autoradiogram (Fig. 3, lane B) showed that only single fragments were visible in blots made with several restriction enzymes: the size of these fragments were as expected from the restriction map for the SAA3 genes (Fig. 2). (Weak hybridization of probe B to the SAA1 or SAA2 genomic fragments was observed in some blots.) We have also analyzed the SJL mouse genomic DNA and found that probe A hybridized to two genomic fragments in Southern blots and probe B to single fragments, though the length of the restriction fragment is polymorphous (*8*). Southern blot hybridization was also carried out in low-stringency conditions [standard saline citrate (SSC) at 50°C] to examine the possibility that other distantly related SAA genes might be present: an identical hybridization pattern was obtained except that very weak hybridization of both probes to a 4.6-kb fragment was observed in some blots made with Eco RI (*9*). A similar hybridization pattern was reported by Taylor and Row (*8*) who used SAA3 cDNA (*6*) as a probe. Thus, these results indicate that the mouse SAA is encoded by at least three genes specific for SAA1, SAA2, and SAA3.

In screening about 16,000 recombinants from a liver cDNA library of lipopolysaccharide (LPS)–stimulated BALB/c mice with a 540-bp Bgl II–Xho I fragment from mouse

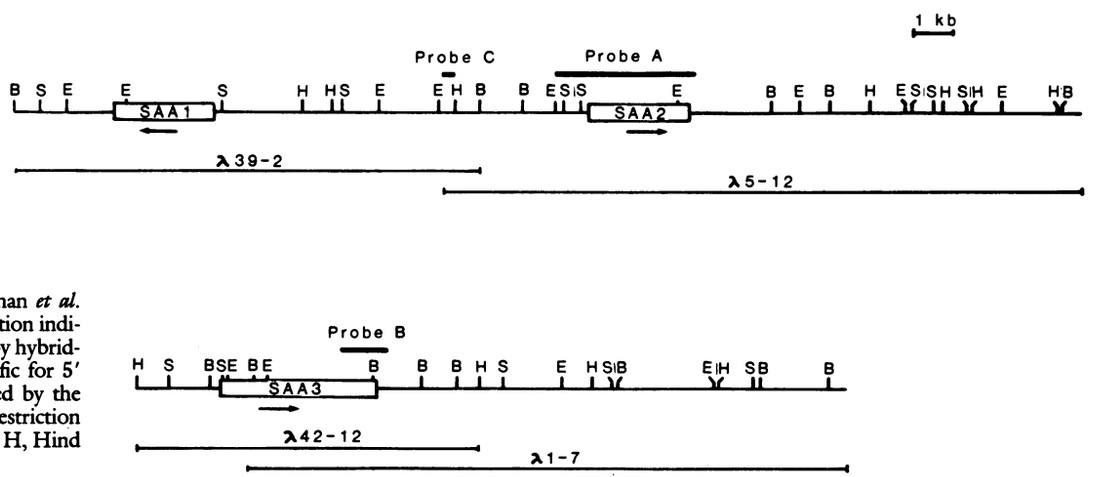

Fig. 2. Restriction maps of the mouse genomic clones containing SAA1, SAA2, and SAA3 genes. The tentative 5′ and 3′ ends (i) for the SAA1 and SAA2 genes were determined from hybridization with a full-length SAA2 cDNA (*3*) and detailed restriction mapping and (ii) for the SAA3 gene, from hybridization with SAA2 cDNA and the results by Stearman *et al.* (*7*). Orientation of gene transcription indicated by arrows was determined by hybridization with cDNA probes specific for 5′ and 3′ ends. The regions covered by the probes are indicated by bars. Restriction enzymes: E, Eco RI; B, Bam HI; H, Hind III; and S, Sac I.

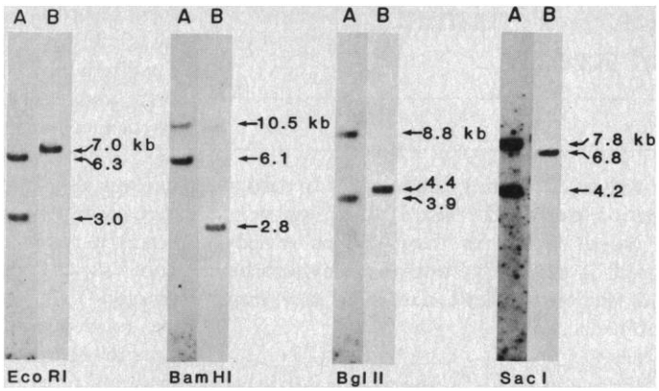

Fig. 3. Southern blot analysis of BALB/c mouse genomic DNA with the SAA2 (probe A in Fig. 2) and SAA3 (probe B in Fig. 2) genomic probes. BALB/c mouse liver DNA (2 μg) (*18*) was digested to completion with restriction enzymes, sized on 0.8 percent agarose gels, transferred to a nitrocellulose filter (*19*), and hybridized with radioactive probes (labeled by nick translation). The filters were washed two times at room temperature with 0.3*M* NaCl and 0.03*M* sodium citrate (2× SSC) and three times at 65°C for 20 minutes with 0.1× SSC and 0.1 percent sodium dodecyl sulfate (SDS) and subjected to autoradiography at −70°C. Blots hybridized with probes A and B are indicated by A and B.

SAA2 cDNA used as a probe, we could identify 8 SAA1 and 25 SAA2 cDNA clones, but no SAA3 cDNA clone. (Colony hybridization was performed under conditions of low stringency.) The SAA3 gene thus seems to be expressed only at a low level during acute phase reaction. To further study the degree of expression of the genes for SAA1, SAA2, and SAA3, we have synthesized 18 base–long oligonucleotide probes (*10*) complementary to the region where the SAA1, SAA2, and SAA3 cDNA sequences diverge (Fig. 1A) (*3*). The oligonucleotide probes were labeled at the 5′ end with T4 polynucleotide kinase in the presence of adenosine-[γ-^{32}P] triphosphate (*11*) and used as hybridization probes in Northern blotting experiments: The specificity of these probes was established in Southern blots analysis of DNA's from SAA1 cDNA, SAA2 cDNA, and SAA3 genomic clones. The SAA1- and SAA2-specific probes strongly hybridized to liver RNA from LPS-stimulated BALB/c mice, though none of these probes hybridized significantly to RNA from normal mouse (Fig. 3B). By contrast, the SAA3-specific probe hybridized only weakly to RNA from LPS-stimulated mouse. Essentially the same pattern of RNA hybridization was observed in blots of RNA from LPS-stimulated Swiss mice. However, in SJL mice, the SAA2-specific probe showed only weak hybridization to LPS-induced RNA, though the SAA1-specific probe hybridized to LPS-induced RNA of SJL mice as strongly as to those of BALB/c and Swiss mice. These results were further confirmed by the results of in vitro translation of liver messenger RNA (mRNA) from LPS-stimulated mice (Fig. 4). As reported previously (*3*), mRNA from LPS-stimulated BALB/c and Swiss mice directed the synthesis of large quantities of two polypeptides with molecular weights of 12.8 and 12.5 kD corresponding to pre-SAA1 and pre-SAA2, respectively. Although the SAA2 band seems to be more intense than the SAA1 band, pre-SAA2 and pre-SAA1 contain four and three methionine residues, respectively (*3*). By contrast, in translation of mRNA from LPS-stimulated SJL mice, the intensity of the SAA2 band reduced substantially, though the intensity of the SAA1 band was comparable to those in BALB/c and Swiss mice. These results indicate that during acute phase reaction both the SAA1 and SAA2 genes are expressed at high levels in BALB/c and Swiss mice (though SAA3 gene expression is barely detectable) and that in SJL mice SAA2 gene expression is very low. One implication of these findings for amyloidogenesis is that the decreased expression of the SAA2 gene in SJL mice is related to the resistance of these mice to azocasein-induced amyloidosis (*12*), since only SAA2-derived products are found in amyloid tissues (*4*). However, the possibility that the resistance to amyloidosis is due to allotypic substitutions in the SAA2 coding sequence cannot be completely ruled out in the absence of data on the SAA2 cDNA sequence in SJL mice.

The presence of more than one form of SAA has also been recognized in other mammals including humans (*2, 13*). Furthermore, there is evidence for the diverse expression of SAA isotypes in individual rabbits during acute phase reaction (*2*). Therefore, it is of interest to examine whether the expression of human SAA isotypes also shows individual variation in various disease states. Such variation would play a critical role in amyloidogenesis, though it is not known whether one human SAA isotype is more amyloidogenic (*1*) than others as in mouse isotypes. Study of the recently isolated cDNA and genomic clones for human SAA (*14*) would shed light on these problems.

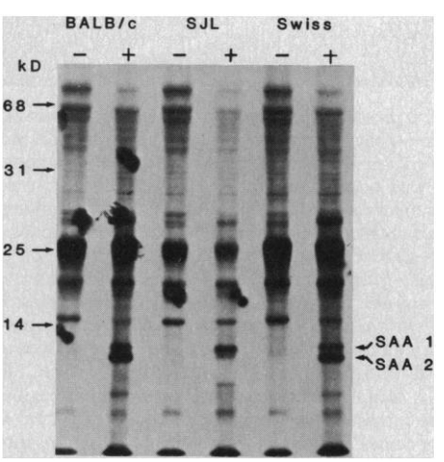

Fig. 4. SDS–polyacrylamide gel electrophoretic analysis of cell-free translation products of liver mRNA from normal (−) and LPS-stimulated (+) mice. Polyadenylated RNA was translated in a wheat germ system (Bethesda Research Laboratories) in the presence of [^{35}S]methionine, and translation products were analyzed on 13 percent slab gels containing 6*M* urea (*17*).

REFERENCES AND NOTES

1. M. B. Pepys and M. L. Baltz, *Adv. Immunol.* **34**, 141 (1983); G. G. Glenner, *N. Engl. J. Med.* **302**, 1283 (1980); *ibid.*, p. 1333.
2. P. S. Tobias, K. P. W. J. McAdam, R. J. Ulevitch, *J. Immunol.* **128**, 1420 (1982).
3. K. Yamamoto and S. Migita, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2915 (1985).
4. J. S. Hoffman, L. H. Ericsson, E. Eriksen, K. A. Walsh, E. P. Benditt, *J. Exp. Med.* **159**, 641 (1984).
5. W. D. Benton and R. W. Davis, *Science* **196**, 180 (1977).
6. J. F. Morrow, R. S. Stearman, C. G. Peltzman, D. A. Potter, *Proc. Natl. Acad. Sci U.S.A.* **78**, 4718 (1981).
7. R. S. Stearman, C. A. Lowell, W. R. Pearson, J. F. Morrow, *Ann. N.Y. Acad. Sci.* **389**, 106 (1982).
8. B. A. Tayor and L. Rowe, *Mol. Gen. Genet.* **195**, 491 (1984).
9. We have recently isolated the cosmide clone that gave rise to a 4.6-kb Eco RI fragment in Southern blot analysis with an SAA2 cDNA probe (unpublished data). This clone probably represents the fourth SAA gene.
10. S. L. Beacage and M. H. Caruthers, *Tetrahedron Lett.* **22**, 1859 (1981).
11. A. M. Maxam and W. Gilbert, *Methods Enzymol.* **65**, 499 (1980).
12. H. Miyamoto, personal communication.
13. N. Eriksen and E. P. Benditt, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6860 (1980); L. L. Bausserman, P. N. Herbert, K. P. W. J. McAdam, *J. Exp. Med.* **152**, 641 (1982).
14. G. H. Sack, Jr., *Gene* **21**, 19 (1983); J. D. Sipe *et al.*, *Biochemistry*, in press.
15. J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **18**, 5294 (1979); H. Aviv and P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1408 (1972).
16. P. S. Thomas, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5201 (1980).
17. V. K. Laemmli, *Nature (London)* **227**, 680 (1970).
18. Y. Yaoita and T. Honjo, *Biomed. Res.* **1**, 164 (1980).
19. E. M. Southern, *J. Mol. Biol.* **98**, 503 (1975).

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