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Journal of Chromatography B, 697 (1997) 181–188

JOURNAL OF
CHROMATOGRAPHY B

Temporal analysis of DNA restriction digests by capillary electrophoresis

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Abstract

We demonstrate a facile means for temporal analysis of DNA restriction enzyme digests by capillary electrophoresis–laser-induced fluorescence (CE–LIF) detection. ϕX-174 DNA was digested with HaeIII restriction enzyme under conditions that allowed the monitoring of digestion as it proceeded toward completion. Separation by a polymer solution of methylcellulose in a polyacrylamide coated capillary allowed high resolution and a high degree of reproducibility between sequential runs. At pre-selected time intervals an injection of the digest, directly from the reaction mixture, was made. Sensitive detection was achieved by using ethidium bromide as an intercalation dye and allowing intercalation to occur on-column. It is demonstrated that the course of the digestion (i.e., the creation and diminishing of fragment peaks) can be followed using this methodology. Also demonstrated is the ability to use temporal analysis to determine ideal conditions for producing a single cut within a cloning and expression vector (pET3a-PAI-1) which contains 11 potential restriction endonuclease cleavage sites. This initial attempt to follow a restriction digest on-column not only provides meaningful information for the biochemical researcher, but also furthers the use of CE as a diagnostic tool for the biochemical laboratory. © 1997 Elsevier Science B.V.

Keywords: DNA

1. Introduction

The unique characteristics inherent to capillary electrophoresis (CE) present novel means to investigate analyses performed by more traditional biochemical methods. Most important among these are speed, efficiency (hence resolving power) and small sample volumes. In fact, CE is already showing widespread success as a biochemical method for the analysis of sequencing [1–3], restriction digest analysis [4–6], protein–DNA interactions [7], selected sections of DNA obtained by the polymerase chain reaction (PCR) [8–11] and others. The ability to exploit the unique characteristics of CE will continue to make it a more robust tool for biochemical analysis.

Studies of DNA restriction digests by CE have typically centered around the use of these samples to adjust and optimize separation conditions that will lead to increased resolution and reproducibility, as well as extremely fast analysis times. A great deal of this work is intended to make CE a more rigorous tool for genome sequencing, where fast analysis times and high reproducibility are extremely important. Researchers are continuously demonstrating how new sieving media (e.g., new polymers or new

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PII S0378-4347(97)00227-2

combinations of polymers) produce greater degrees of resolution. Also new capillary coating procedures, and even the absence of column coating combined with certain polymeric matrices produce excellent run-to-run reproducibility. In another illustration of the utility of this technique, we have chosen to investigate CE as a diagnostic tool for the biochemical researcher by exploiting its advantages for the temporal analysis of DNA restriction digests.

DNA restriction enzyme digests generate sets of fragments resulting from the enzyme cutting the double stranded DNA at specific sequence sites. During the digestion, the enzyme cleaves particular phosphodiester linkages within the DNA as it comes in contact with the specific sequences of base pair (bp) sites that it recognizes. As the digestion progresses towards completion, large fragments are further recognized and digested into smaller fragments until all restriction sites are cleaved. The size of the resulting fragments, in bp number, is usually known. However, a certain fragment that may be required for further research may not be generated from a complete digestion. Having access to these incompletely digested fragments and being able to determine the time during the digestion when these fragments are generated provides the researcher a greater range of DNA fragments to utilize for biochemical research.

Biochemical researchers traditionally approach this problem by performing partial DNA restriction digests. These partial digests serve the purpose of generating specific DNA fragments that are not available from digesting the DNA to completion with commercially available restriction enzymes. Once it is known the time point during the digestion when a certain sized fragment is generated, the fragment can then be isolated for cloning and/or other purposes. At certain times during the digestion, the reaction is terminated resulting in a partially digested mixture that contains fragments that are different from those that result from complete digestion. This is typically achieved by terminating the digest via heat inactivation or phenol addition/extraction. The analysis of partial digests is traditionally performed by slab gel electrophoresis. The size of the fragments is determined by comparison of the migration distances to a standard containing DNA fragments of known size. However, in many cases, gel conditions cannot be easily optimized to produce the resolution that is necessary to determine the presence of a partial digest fragment. A problem with analyzing partial digests by slab gel electrophoresis is the lack of resolution inherent to agarose gels. Also, because it is not known prior to the analysis when a certain size fragment is present, the procedure must be performed numerous times, varying digestion times and amounts of restriction enzyme in the reaction mixture. The entire process can be extremely costly and time consuming, requiring multiple analyses performed to arrive at the desired information empirically.

An alternative approach involves using the high resolving power, speed and small sample requirements of CE as a diagnostic tool to monitor the time course of the digestion. As illustrated herein, a restriction digest can be performed under any desirable conditions and injected directly onto the capillary column, eliminating both the need to terminate the digestion for the purpose of sampling and any clean-up step that may also be needed. The ability to flush the column between runs allows the introduction of fresh sieving matrix, effectively eliminating carryover effects from the previous injection (most importantly the enzyme used in the digestion reaction). We present herein a temporal analysis of the ϕX-174 HaeIII DNA digest. It is demonstrated that the creation and diminishing of fragments peaks can be followed as the digestion progresses towards completion. Also demonstrated is the use of a temporal analysis by CE to determine when a cloning and expression vector containing a gene of interest (pET3a-PAI-1) has been linearized by the BstYI restriction enzyme, an enzyme known to have 11 cut sites on this vector.

A partial digest of the pET3a-PAI-1 DNA with the restriction enzyme BstYI is desired for subsequent cloning of the DNA fragment which contains the cDNA encoding PAI-1 into another plasmid vector. The digestion strategy required a partial digest with BstYI to avoid fragmentation of the coding sequence at an internal BstYI restriction site. As a first step in the cloning, a limited BstYI digest is desired to produce cut plasmid with an average of one cut per molecule at any of the potential 11 cleavage sites. Subsequent digestion with a second enzyme, NdeI, cleaves at a unique site within the pET3a-PAI-1

plasmid at a position which lies immediately upstream of the coding sequence for PAI-1. One of the several NdeI/BstYI fragments produced will be approximately 1350 bp in length and will contain the intact cDNA for PAI-1. Ultimately, this approximately 1350 bp fragment from the NdeI/BstYI double digestion will be isolated from the digestion mixture and sub-cloned into another plasmid vector. The immediate goal of this work was to use CE to rapidly and conveniently evaluate the temporal framework of the initial BstYI digestion step to determine conditions which produce predominantly single-cut plasmid. CE has distinct advantage over agarose electrophoresis in its ability to detect low-molecular-mass fragments that will not be observed in the slab gel.

2. Experimental

2.1. Materials

Tris(hydroxymethyl)aminomethane, boric acid and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma (St. Louis, MO, USA) and used to prepare pH 8.5 Tris–boric acid–EDTA buffers (TBE). Methylcellulose (MC: 4000 cp for 2.0%, w/v), ethidium bromide (EB), acrylamide, ammonium persulfate (APS), N,N,N′,N′-tetramethylethylenediamine (TEMED) and γ-methacryloxypropyltrimethoxysilane (γ-MTMS) were also obtained from Sigma. Samples of ϕX-174 DNA HaeIII digest (390 μg ml^{-1}), ϕX-174 phage DNA (10 u ml^{-1}), HaeIII restriction enzyme (14 u μl^{-1}) with incubation buffer and a 50 bp step ladder (0.25 μg μl^{-1}) were obtained from Sigma. pET3a-PAI-1 DNA (0.7 μg ml^{-1}) was the kind gift of Dr. David Ginsburg, Howard Hughes Medical Institute, University of Michigan Medical Center (Ann Arbor, MI, USA). BstYI restriction enzyme (10 000 u/ml) with incubation buffer was obtained from New England Biolabs (Beverly, MA, USA). Fused-silica capillaries (50 and 75 μm I.D.×365 μm O.D.) were obtained from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Column preparation

Silica capillaries with lengths of 60 cm (50 cm to the detection window) were coated with linear polyacrylamide using the following modified version of a procedure described by Hjerten [12]. The capillaries, prepared in duplicate, were sequentially washed with 5 *M* NaOH for 1 h, water for 10 min, 5 *M* HCl for 1 h and water again for 10 min. The capillaries were then washed for 10 min with acetone followed by reaction with a solution of acetone–γ-MTMS (1:1, v/v) for 24 h. Excess γ-MTMS was removed by flushing the capillary with water for 10 min. The γ-MTMS was reacted in-situ with linear polyacrylamide. A fresh solution of acrylamide was prepared by adding 0.4 g of acrylamide to 9 ml of 50 m*M* K_2HPO_4, buffered to pH 6.8. A 67 m*M* solution of ammonium persulfate was also prepared and both solutions were degassed by bubbling helium through them for 30 min. The polymerization reaction was initiated by adding 1 ml of the APS solution, spiked with 7.5 μl TEMED to the acrylamide solution. The resulting polyacrylamide was pushed through the columns and allowed to react for 20–24 h. Unreacted polyacrylamide was removed by flushing the capillary with 10 m*M* H_3PO_4. Columns were stored and rinsed between runs with 10 m*M* H_3PO_4.

2.3. Running buffer and sample preparation

All running buffers used in this work contained 45 m*M* TBE with the appropriate percentage of MC. These methylcellulose containing buffers were prepared in a manner previously described [13]. Digestion conditions for the HaeIII digestion of ϕX-174 DNA and the NdeI digestion of pET3a-PAI-1 DNA consisted of 1 μl of DNA, 2 μl of the appropriate incubation buffer, 17 μl H_2O and 0.5 μl and 1 μl restriction enzyme, respectively. Digestion conditions for the BstYI digestion of pET3a-PAI-1 consisted of 1 μl of DNA, 2 μl incubation buffer, 17 μl H_2O and the appropriate amount of diluted restriction enzyme in a total reaction volume of 20 μl.

2.4. Apparatus and electrophoresis

The electrophoresis and fluorescence apparatus was assembled in-laboratory and is described in detail in a prior report [13]. A PMS Electro-Optic helium neon (Edmund Scientific, Barrington, NJ, USA) "grennie" laser (1.5 mW at 543.5 nm) in conjunction with a 25 mm f/1 lens served as the

excitation source, while a 1 mm core diameter quartz fiber optic provided collection of the EB intercalated fluorescence emission. The emission was isolated using a 570 nm cut-on filter and monitored using a Hamamatsu (Bridgewater, NJ, USA) R282 PMT and a Pacific Precision Instruments (Concord, CA, USA) Model 126 photometer. Electropherograms were recorded using a data collection routine and computer programmed in-laboratory [14] and a strip chart recorder. A Hipotronics (Brewster, NY, USA) Model 840A high-voltage power supply was used to apply electrophoretic fields and injections were performed electrokinetically.

3. Results and discussion

To obtain the best separation conditions for DNA fragments in CE, it is important to be able to optimize for those conditions which are the most important for a given separation situation [15]. When considering DNA restriction fragment digests important considerations may include resolution of fragment peaks, efficiency, reproducibility and analysis time. The degree to which these considerations effect the separation is ultimately dependent upon what information is warranted from the separation. In a large number of restriction digest separations, resolution of the two or three most difficult to resolve fragments may be the main goal, while in other cases the identification of each fragment is all that is necessary, leaving resolution to be sacrificed to some degree.

When attempting to follow the time course of a restriction digest to completion other considerations become more important. Since this technique deals with the injection of the digest directly from the reaction mixture, analysis time is extremely important. The greater the number of injections that can be made during the course of the digestion the greater the sampling rate, allowing a more accurate determination of when certain fragments begin to appear and disappear. If CE conditions are such that an analysis takes 30 min to complete, generally only a couple of time points can be analyzed before the digestion is complete, yielding a potentially poor overview of the digestion course.

Using a computer program developed in our laboratory we are able to optimize separation conditions for the fast separation of restriction digests [15]. Taking into consideration excess and inadequate resolution of fragments and analysis time, conditions can be selected, prior to actual experimentation, that will result in the best separation in the shortest amount of time. This advantage allows us to perform experiments sequentially every 10–12 min, allowing considerably more time points to be experimentally determined during the course of the digestion. This procedure allows us to bypass the many time consuming steps required for slab gel analysis. The speed of CE runs allows us time between runs to rinse the column (with dilute acid) and fill it with fresh sieving matrix. This step helps to overcome any carryover effects from the previous runs. We are able to inject directly from the digestion reaction mixture without being concerned with the deleterious effects of the restriction enzyme resulting in near real-time analysis of digestions.

It can be seen in Fig. 1 that the progression of a restriction digest can be followed by CE. ϕX-174 plasmid DNA is digested with HaeIII restriction enzyme under conditions that allow the digestion to progress at a slower rate than under normal circumstances. Digestion conditions were based upon manufacturers suggestions with the exception of incubation temperature and concentration of restriction enzyme. The digestion was carried out on ice as opposed to 37°C and the normal amount of enzyme used was halved. It can be seen that at approximately 6 min (see t=6 min separation in Fig. 1) into the digestion a single prominent fragment peak is evident relatively late in the electropherogram. Comparison of the migration time of this peak (11.3 min) to that of the last eluting peak of the 50 bp step ladder (3147 bp, 11 min, Fig. 1) leads us to believe that this peak corresponds to a single cut resulting in the linearization of the ϕX-174 plasmid DNA (5386 bp).

A closer look at the 6 min run shows evidence of additional fragmentation prior to the 11 min fragment. After approximately 26 min into the digestion a significant amount of digestion has occurred. Comparison of migration times to that of the completed digest (seen at t=118 min, Fig. 1), where the elution order is 72, 118, 194, 234, 271, 281, 310, 603, 872, 1078 and 1353 bps, it is evident that the 72

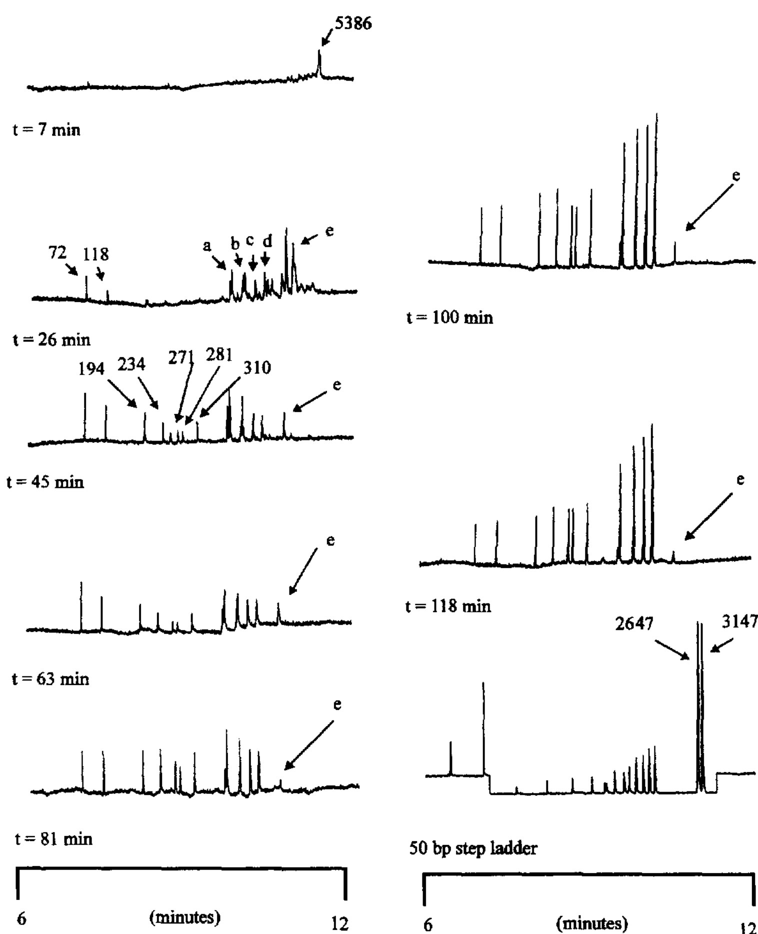

Fig. 1. Electropherogram depicting temporal analysis of ϕX-174 HaeIII DNA digest. Sampling times are labeled directly below each representative electropherogram. See Section 3 for figure depictions. Conditions: 75 μm column, 45 m*M* TBE, 0.5% methylcellulose, pH 8.0, running voltage: 400 V/cm.

and 118 bp fragments are beginning to appear. Also apparent is the disappearance of the 11.3 min fragment. The last four migrating peaks of the digest, 603, 872, 1078 and 1353, are also becoming evident late in the elution window, labeled a, b, c and d, respectively. However it appears as though each of the latest eluting peaks in the 26 min sampling has a significant shoulder indicating the presence of partial fragments that are not present upon complete digestion.

Sampling the digest at approximately 45 min shows that the digestion is beginning to appear more like the completely digested sample. The 72 and 118 fragments are becoming more intense and there is evidence of the mid-eluting fragments (194, 234, 271, 281 and 310). If the time course of the digestion is followed to completion it is observed that as the shoulder appearing on the 603 fragment (a) decreases over time, the intensity of the 271 and 281 fragment peaks increases. It is likely that this shoulder is actually a 552 bp fragment and its digestion produces the 271 and 281 bp fragments. This trend can be followed visually as the digestion progress through the 45, 63, 81, 100 and 118 min samplings.

The fragment peak labeled (e) is seen in each of the samplings at a migration time of approximately

10.8 min. When this peak is followed through the progression of the digestion it appears to be diminishing in intensity as the 1078 (c) and 1353 (d) fragment peaks increase in intensity. Comparison of the migration time of this peak (10.8 min) to that of the second to last eluting peak of the 50 bp step ladder (2647, 10.9 min) leads us to believe that this peak corresponds to a 2432 bp fragment (the partial fragment leading to the 1078 and 1353 bp fragments).

This ability to follow the time course of a restriction digest can prove to be a very time saving methodology for the biochemical researcher as well as providing interesting bioanalytical information about the progression of the digest itself. While it may prove difficult to repeat exact sampling times from digestion to digestion, three separate digests of the ϕX-174 plasmid DNA yielded highly reproducible results in terms of the digestion trends discussed above. Intra-run pooled standard deviations (clearly identifiable peaks) averaged 2.7 s ($n=5$–6) while the inter-run pooled standard deviation was 4.2 s ($n=3$). While run to run reproducibility is important, an advantage inherent to restriction digest analysis is that there are generally recognizable fragment patterns. This relaxes the need for extreme precision (i.e., the standard deviations obtained herein are more than adequate). While the digestion of ϕX-174 plasmid DNA may not prove to be a challenging separation, it did allow us to set conditions for temporal digests of other potentially more important DNA digestions.

Partial digestions in the biochemical laboratory are often required in recombinant DNA work to avoid fragmentation of a particular DNA sequence of interest. An example of such a partial digestion strategy is given with the limited BstYI digest of pET3a-PAI-1 which will avoid cleavage of the cDNA encoding PAI-1 at an internal BstYI site. An experimental approach which yields plasmid cut at a single site is required as an initial step to isolate an intact coding sequence from pET3a-PAI-1.

Fig. 2 shows the ability of temporal analysis by CE to determine ideal conditions for producing a single cut of the cloning vector pET3a-PAI-1. pET3a-PAI-1 DNA is a 5954 bp cloning vector that contains the 1206 bp encoding gene plasminogen activator inhibitor-1 (PAI-1). Complete digestion of this DNA vector with the BstYI restriction enzyme yields 10 fragments: 11, 12, 17, 86, 471, 768, 863, 979, 1298 and 1449 bps in length.

The first electropherogram in Fig. 2 shows the digestion of pET3a-PAI1 with NdeI, a restriction enzyme known to produce a single cut in this cloning vector. A single fragment, 5954 bp, has a migration time of 12 min. This digestion was used simply as a reference point for the migration time of a single cut species. The goal of this series of experiments was to determine what conditions produce a single cut with the restriction enzyme BstYI. Digestion conditions were based upon manufacturers suggestions with the exception of concentration of restriction enzyme. The digestion was carried out at 60°C and the amount of enzyme used was adjusted appropriately.

A 1:10 dilution of enzyme was initially investigated. At 5 min into the digestion a considerable amount of digestion is already occurring (see Fig. 2, $t=5$ min). It is evident however that the most intense peak at this time is still the peak that corresponds to a single cut appearing at 12 min. Further sampling of the 1:10 digest shows an envelope of fragments, signifying that the digestion is still incomplete. It is evident that a 1:10 dilution of BstYI is too concentrated to produce a single cut under these experimental conditions.

A 1:100 dilution of enzyme was investigated next. At 5 min into the digestion, a faint peak is seen at approximately 12 min. This signifies that this concentration of BstYI may be the appropriate amount to produce a single cut. 25 min into this digestion it becomes more evident that a single cut is achieved. Following the digestion out to 52 min begins to show a small envelope of fragmentation prior to the single cut species. A continuation of the 52 min electropherogram shows undigested DNA appearing at approximately 18 min (as compared to an injection of uncut DNA not shown). This series of experiments has shown that a 1:100 dilution of BstYI will produce a single cut when digested in under 20 min. This entire series of experiments was conducted in under 3 h, including analysis time. The more traditional approach to evaluate the partial digestion conditions using agarose gel electrophoresis gave similar results indicating that a dilution of enzyme from 1:100 to 1:500 was necessary to achieve a single-cut plasmid. In contrast to the rapid analysis

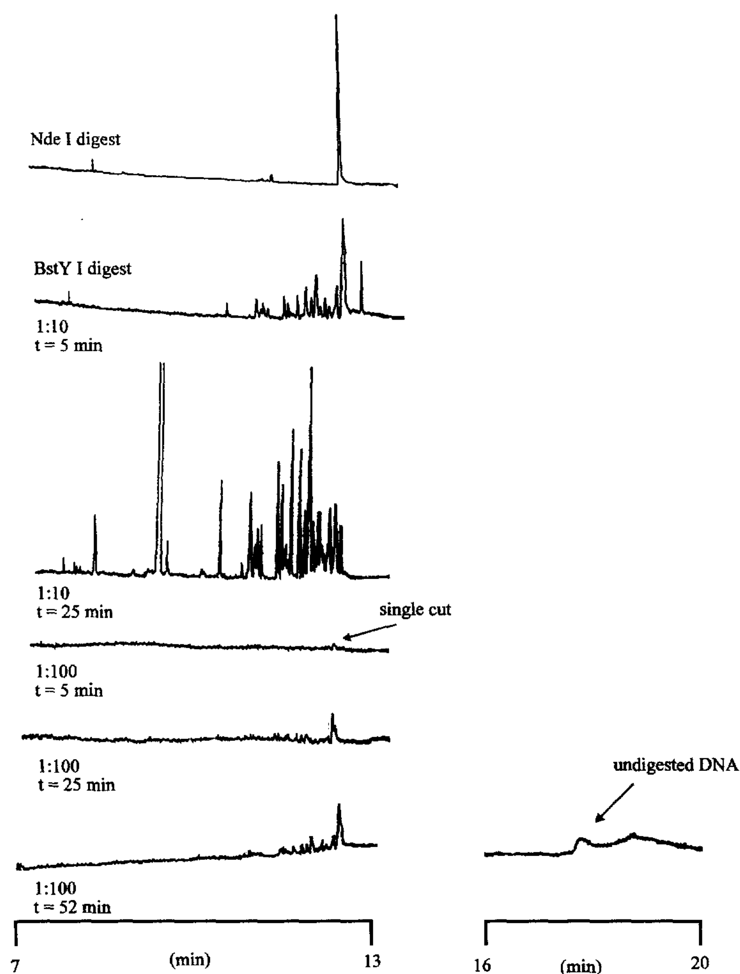

Fig. 2. Electropherogram depicting temporal analysis of pET3a-PAI-1 DNA. The top electropherogram is a single cut digest with NdeI. The subsequent electropherograms represent digestions with BstYI. Sampling times and enzyme concentration are labeled directly below each representative electropherogram. See Section 3 for details. Conditions: 50 μm column, 45 m*M* TBE, 0.5% methylcellulose, pH 8.0, running voltage: 400 V/cm.

time afforded by CE, the use of slab gels to evaluate the digestion conditions required several days and many more test reactions.

Acknowledgments

This work was sponsored by the Division of Chemical Sciences, Office of Basic Research, United States Department of Energy, under grant DE-FGO2-96ER14609 with the University of Tennessee, Knoxville (M.J.S.) and the Heart, Lung and Blood Institute of the National Institutes of Health, under grant HL50676 with the University of Tennessee, Knoxville (C.B.P.). Support was also contributed by The Procter and Gamble Company, Merck and Company, Pfizer Central Research (M.J.S.) and by an Established Investigator Award from the American Heart Association (C.B.P.).

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