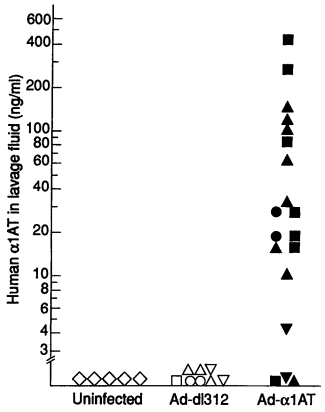

Fig. 5. The amount of human α1AT in the respiratory ELF of cotton rats after in vivo infection with Ad-α1AT. Animals were infected intratracheally with CsCl-purified Ad-α1AT (10^8 to 10^{10} PFU/ml) as described in Fig. 3; controls included uninfected animals and those infected with a similar titer of Ad-dl312. From 1 to 7 days after infection, ELF was obtained by lavage of the lungs with 2 ml of PBS. Lavage fluid was clarified (700*g*, 20 min), and the concentration of human α1AT was quantified (in quadruplicate) with a human α1AT–specific ELISA (*35*) with a sensitivity of ≥3 ng/ml. Each symbol represents the mean value of an individual animal. All uninfected animals, ◇; for infected animals, 1 (○, ●), 2 (△, ▲), 3 (□, ■), and 7 (▼, ▽) days after infection, respectively. No α1AT was detected by ELISA in the viral preparations used for infection.

ined in detail. Safety is particularly important in weighing risk and benefit in response to α1AT deficiency, in which augmentation therapy with human plasma α1AT is available (*21*). In contrast, no definitive therapy is available for CF. Most human adults have antibodies to one of the three serogroup C adenoviruses to which Ad5 belongs (*5*). This implies little risk to those working with these vectors but may have negative implications for the virus as a gene transfer vector in the human lung. If such problems are encountered, alterations in the vector construct may be helpful. The encouraging results obtained with the Ad-α1AT recombinant adenoviral vector in vivo suggest that similar recombinant vectors may be useful for in vivo experimental animal studies with genes such as the human CF gene.

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36. We thank B. Trapnell and N. McElvaney (Pulmonary Branch, National Heart, Lung, and Blood Institute), M. Courtney (Research Department, Delta Biotechnology, Ltd.), B. Carter (Laboratory of Molecular and Cellular Biology, National Institute of Diabetes and Digestive and Kidney Diseases), and H. Ginsberg (Departments of Medicine and Microbiology, Columbia University) for helpful discussions; G. Prince and M. Redington (Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases) for making cotton rats available to us; and T. Raymer for assistance in preparing the manuscript.

16 November 1990; accepted 5 March 1991

Group I Intron Self-Splicing with Adenosine: Evidence for a Single Nucleoside-Binding Site

MICHAEL D. BEEN* AND ANNE T. PERROTTA

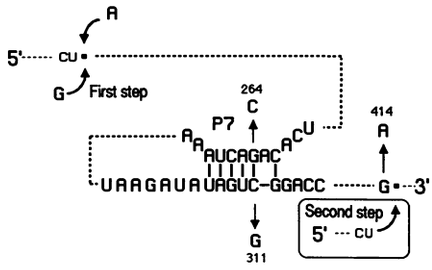
For self-splicing of *Tetrahymena* ribosomal RNA precursor, guanosine binding is required for 5′ splice-site cleavage and exon ligation. Whether these two reactions use the same or different guanosine-binding sites has been debated. A double mutation in a previously identified guanosine-binding site within the intron resulted in preference for adenosine (or adenosine triphosphate) as the substrate for cleavage at the 5′ splice site. However, splicing was blocked in the exon ligation step. Blockage was reversed by a change from guanine to adenine at the 3′ splice site. These results indicate that a single determinant specifies nucleoside binding for both steps of splicing. Furthermore, it suggests that RNA could form an active site specific for adenosine triphosphate.

GROUP I INTRONS SHARE CONserved sequence elements and a common core secondary structure (*1*). Consistent with these similarities, there is a common mechanism by which group I introns are excised and the exons ligated (*2, 3*). A significant feature of all group I introns is the requirement for G (*4*) to initiate the splicing reaction (*2, 5*). The first step is a G-dependent cleavage at the 5′ splice site. This step is a transesterification (phosphoester transfer) reaction in which the substrate G is covalently joined to the 5′ end of the intron. As a result, a free 3′ hydroxyl group is generated on the U at the end of the 5′ exon. In the second step of the splicing reaction, the exons are ligated by another transesterification reaction. In this case, attack occurs at the 3′ side of the conserved G at the 3′ end of the intron (G414) (*6*) (Fig. 1). This G is essential for completion of the splicing reaction (*7*). The 5′ splice-site cleavage can be viewed as the "forward" reaction, and the exon ligation can be viewed as the "reverse" of the same reaction (*8–10*). This idea was demonstrated

Department of Biochemistry, Duke University Medical Center, Durham, NC 27710.

*To whom correspondence should be addressed.

Fig. 1. Steps in the splicing of group I introns and base changes introduced to the *Tetrahymena* intron sequence. The 5′ and 3′ splice sites are indicated by the small solid squares, the dashed lines represent additional exon and intron sequences, and the sequences for P7 and the flanking regions are shown. The first step, G (or A) attack at the 5′ splice site, is represented by the curved arrows; the second step is indicated by the inset. The positions of the base changes described in the text are denoted with the straight arrows.



with short oligoribonucleotide substrates and a ribozyme derived from the *Tetrahymena* group I intron (*10*). The ribozyme catalyzes both the attack by G on the phosphodiester bond 3′ of CU and the attack by CU on the phosphodiester bond 3′ of G.

The requirement for G in both splicing steps and the similarities between the two steps have resulted in contending models of the ribozyme active site. In one model it was postulated that a single G-binding site first binds and positions the substrate G for attack on the 5′ splice site and subsequently positions G414 for exon ligation (*8, 10*). However, a model with two G-binding sites (*11*) is consistent with the ability of certain group I introns to catalyze G exchange reactions (*7, 11, 12*).

Michel and co-workers (*13*) identified G264 in the conserved pairing, P7, as a base involved in G binding for the first step in splicing (Fig. 1). When the G264 is changed to an A and its presumed pairing partner, C311, is changed to a U, the A:U variant thus generated shows preference, in a 5′ splice-site cleavage assay, for 2-aminopurine ribonucleoside (r2AP). Because the change at position 311 is not required for the altered specificity, G264 is established as a primary determinant for G binding (*13*). With respect to the second step, the A:U variant distinguished poorly between an A and a G at position 414, implicating the same nucleoside-binding site in 3′ splice-site selection.

A critical test of the single G-binding site model would be to substitute r2AP for G414 at the 3′ splice site and to evaluate its effect on exon ligation. Because this is not feasible with current technology, Michel and co-workers (*13*) examined an intermolecular version of the ligation step with 3′ splice-site analogs that consisted of oligonucleotides in which the 5′ terminal nucleotide was either G or r2AP. The oligonucleotide terminating with r2AP was a substrate only for ligation by the A:U variant intron, suggesting that there is indeed a single binding site. However, the assay does not distinguish between the reverse of 5′ splice-site cleavage and exon ligation. In addition, because r2AP is normally not found in RNA, the possibility that group I introns could use other naturally occurring nucleosides in the splicing reaction has not been addressed.

We have generated additional base changes at positions 264 and 311 in the *Tetrahymena* group I intron and have surveyed the splicing activity of the variant precursors with other substrates (*14*). A C:G variant precursor, a mutant with a G to C change at position 264 and a C to G change at position 311 (G264C:C311G) (Fig. 1), produced little or no ligated exon (Fig. 2, lanes 11 to 15). Although incubation of this precursor in a solution that contained Mg^{2+} resulted in hydrolysis at the 5′ splice site as shown by the presence of free 5′ exons and introns ligated to 3′ exons (lane 12) (*15*), the addition of guanosine triphosphate (GTP) (0.5 mM) (lane 13) or G (0.2 mM) (*16*) did not increase the amount of free 5′ exon. In contrast, there was enhanced cleavage at the 5′ splice site with A and purine ribonucleoside (rP) (lanes 14 and 15, respectively). However, no excised intron or ligated exons were produced. Precursors containing the wild-type (wt) intron have been shown to react with adenosine triphosphate (ATP) (*8*) although they are 10^4 to 10^5 times more reactive with GTP (*8*). Because of the high concentrations of nucleosides used in these experiments, considerable splicing of the wt sequence occurred in 30 min with A (lane 4) and ATP (*16, 17*), but there was little activity with rP (lane 5). Cleavage at the 5′ splice site of the C:G variant precursor was also enhanced by ATP, but not by cytidine triphosphate, uridine triphosphate, inosine, 6-mercaptopurine ribonucleoside, xanthosine monophosphate, or 2-amino-6-chloropurine ribonucleoside (*16*).

Attack by G at the 5′ splice site of precursor RNA containing wt intron sequences results in G being covalently attached to the 5′ end of the intron. Covalent addition of A or rP to the 5′ end of the C:G variant intron and a qualitative assessment of the variant's altered specificity were demonstrated in a mixed substrate experiment. Precursor RNAs were incubated under splicing conditions in mixtures of either G and A or G and rP. The products were then 5′ end-labeled with ^{32}P and the 5′ terminal nucleotide identified (Fig. 3). Only those cleavage products generated by nucleoside addition should be labeled efficiently because intron catalyzed hydrolysis generates a 5′ phosphate group (*8*). Splicing of wt precursor generated only 5′ ^{32}P-labeled guanosine monophosphate (GMP) (lanes 2 and 4). However, splicing of the C:G variant generated 5′ ^{32}P-labeled adenosine monophosphate (AMP) and 5′ ^{32}P-labeled purine ribonucleoside monophosphate (rPMP) (lanes 3 and 5). These results demonstrate that (i) the reaction of the C:G variant with A and rP was not an artifact due to contamination of those nucleosides, (ii) both A and rP were linked to the intron through a 3′,5′-phosphodiester bond, and (iii) if there was no strong bias in the 5′ labeling by polynucleotide kinase, the discrimination

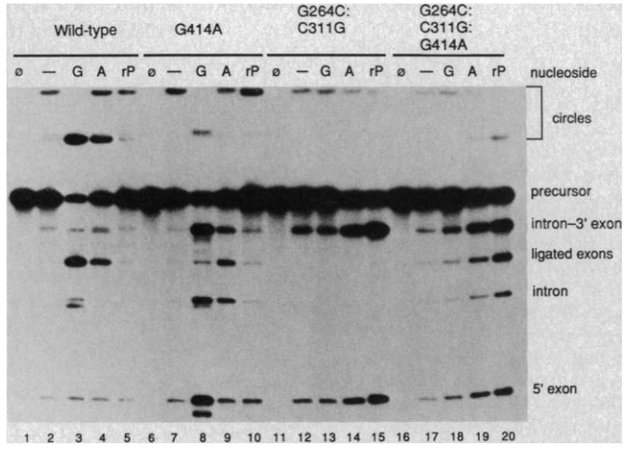


Fig. 2. Cleavage and splicing of wt and variant precursors in the absence or presence of GTP, A, or rP. Precursor RNAs (runoff transcripts internally labeled with ^{32}P and digested with Bam HI) were incubated at 40°C in 50 mM tris-HCl (pH 7.0), 1 mM EDTA, and 0.2 M NaCl either in the absence of Mg^{2+} (∅) or in the presence of 11 mM $MgCl_2$ (all other lanes). The reactions that contained Mg^{2+} either had no substrate nucleoside (−) or contained 0.5 mM GTP (G), 0.5 mM A, or 0.5 mM rP. Reactions (in volumes of 10 μl) were terminated after 30 min with the addition of an equal volume of 95% formamide in 25 mM EDTA. Samples (10 μl) were run on a 5% polyacrylamide gel that contained 7 M urea, 50 mM tris-borate (pH 8.4), and 0.5 mM EDTA. An x-ray film was exposed to the dried gel. The band located just below the 5′ exon band in lane 8 has been identified as a 3′ exon with an extra GTP added to the 5′ end (*16*).

against G by the C:G variant in reactions with mixed substrates was similar to the discrimination against A and rP by the wt intron.

The reaction of the C:G variant with A or rP results in production of 5′ exon and intron–3′ exon reaction intermediates, but no ligated exons (Fig. 2, lanes 11 to 15). This observation suggests that the splicing reaction is blocked after the first step because of an inability of the C:G variant intron to either bind or activate the 3′ splice site. This idea is consistent with a single binding site model, which predicts that the intron should no longer bind G414. If the single binding site model is correct, changing G414 to an A should restore exon ligation. The G414A change was made with both the wt and C:G variant. In the wt background, the G414A change resulted in an increased accumulation of 5′ exon and intron–3′ exon intermediates when incubated with G (Fig. 2, lane 8). This same change in the C:G variant resulted in the production of both the ligated exons and free intron when incubated with A or rP (lanes 19 and 20). The accuracy of the ligation step was established by dideoxy sequencing of isolated ligated exons with reverse transcriptase (*16*).

Splicing of the C:G variants with and without the G414A change was examined in a time course experiment (Fig. 4.) Without the G414A change and in the absence of A, 5′ exon and intron–3′ exon intermediates accumulated over the course of the reaction (30 min) (lanes 1 to 5). Addition of A increased the rate of cleavage at the 5′ splice site five to ten times, but no ligated exons were generated (lanes 6 to 10). However, with the introduction of the G414A change, the intermediates reached steady state after about 10 to 15 min, and ligated exons and introns (linear and circular forms) accumulated (lanes 11 to 15). Without exogenous A, ligated exon was generated at very low levels and was only observed after 30 min (lanes 16 to 20) (*15*).

Using truncated versions of the C:G variant precursors (Sca I runoffs) (*14*), we found that the rates of cleavage at the 5′ splice site reached a plateau at high concentrations of A, ATP, and rP (*16*); this observation could indicate a change in the rate-limiting step or the saturation of a binding site. The C:G variant with rP and A is substantially less active than is the wt with G; the catalytic efficiency, V_{max}/K_m (the maximum cleavage velocity divided by the Michaelis constant, for the C:G variant with rP and A was 1/130 and 1/600, respectively, of the same value for the wt sequence with G (*18*). In comparison, the values for the A:U variant with the substrates r2AP and 2,6-diaminopurine ribonucleoside were about 1/10 and 1/100, respectively, of the value for the wt sequence with G (*13*).

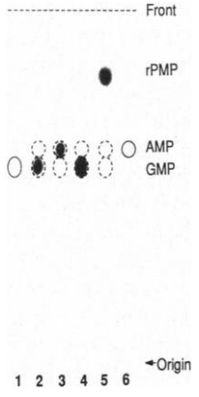


Fig. 3. Identification of the nucleoside added to the 5′ ends of wt and C:G variant intron RNAs. Unlabeled precursor RNAs (wt, lanes 2 and 4; C:G variant, lanes 3 and 5) were incubated under splicing conditions [50 mM tris-HCl (pH 7.0), 11 mM $MgCl_2$, 1 mM EDTA, and 200 mM NaCl, 40°C] either with G and A (0.2 mM each, lanes 2 and 3) or with G and rP (0.2 mM each, lanes 4 and 5). After 30 min, we stopped the reactions with EDTA and precipitated the RNA with ethanol. We then resuspended the RNA in distilled water and labeled the 5′ end of the RNA with ^{32}P on ice; final conditions were 100 mM tris-HCl (pH 8.9), 10 mM $MgCl_2$, 5 mM dithiothreitol, 2.5 μM [γ-^{32}P]ATP (7000 Ci/mmol), and 15 units of T4 polynucleotide kinase. After 2 hours on ice, EDTA was added to the reaction mixture to a final concentration of 25 mM, and formamide was added to a final concentration of 50%. The products were fractionated on a 5% polyacrylamide gel under denaturing conditions as described for Fig. 2. The labeled products (intron for the wt and intron–3′ exon intermediate for the C:G variant) were located by autoradiography and excised. RNA was eluted from the gel slice, ethanol-precipitated, and resuspended in distilled water. We quantified the radioactivity by counting Cherenkov scintillation, and each sample (20,000 cpm) was digested with nuclease P1 (0.2 unit in 4 μl, 50°C, 60 min). We fractionated the products on polyethyleneimine cellulose thin layer plates (EM Science, Cherry Hill, New Jersey) using 1 M LiCl as the mobile phase. Lanes 1 to 5 contained a GMP marker and lanes 2 to 6 had an AMP marker. Both were visualized under ultraviolet light, and their positions are indicated. Although there was no marker for 5′ rPMP, chromatography in a second dimension clearly resolved the labeled rPMP from cytidine 5′-monophosphate and uridine 5′-monophosphate markers.

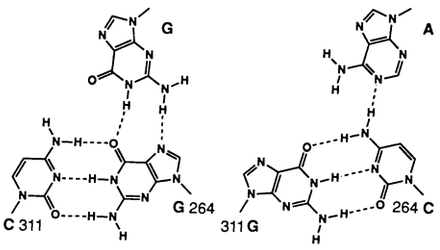


Fig. 5. Models that compare the proposed G-binding site (left panel) and a similar structure for the A-binding mutant (right panel). The G-binding site is the previously proposed structure (*13*).

A model for G binding (*13*) that is consistent with r2AP binding to the A:U variant suggests that either G or r2AP can bind in the major groove of P7, forming triplets involving the free G and the G:C base pair (Fig. 5) or involving the r2AP and the A:U base pair. In both cases the incoming nucleoside is capable of forming two hydrogen bonds to the purine at position 264. According to this model, C264 in the C:G variant may interact with the substrate nucleoside. The potential exists for only one hydrogen bond to form between N4 of C264 and N1 of the incoming A or rP (Fig. 5). Selectivity of the C:G variant, however, could be due to exclusion of G by steric hindrance between hydrogens at the N1 position of the free G and N4 of C264.

Our results support a model in which a single G-binding site is used for both steps of the splicing reaction. The possibility remains that there is a second G-binding site that is not involved in the splicing reaction but that can function in G exchange. Another possibility is that there are two overlapping nucleoside-binding sites that somehow share the feature that determines specificity. This variation on a single binding site model addresses the observation that the binding of G414 may have properties distinct from

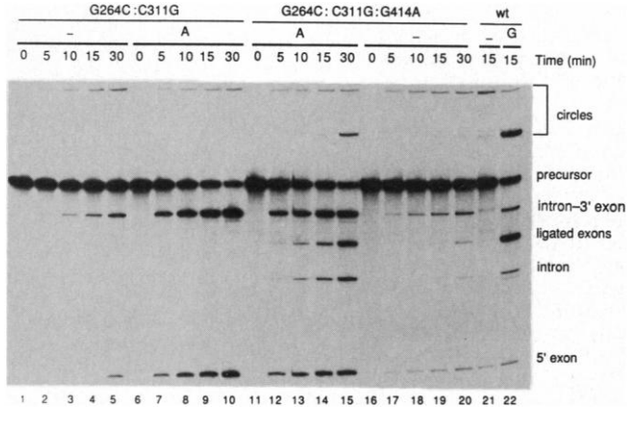


Fig. 4. Time course for cleavage and splicing of the C:G variant precursor with and without the G414A change at the 3′ splice site. Precursor RNAs internally labeled with ^{32}P were incubated in 50 mM tris-HCl (pH 7.0), 1 mM EDTA, and 200 mM NaCl without A (–) or with A (2 mM) for 5 min at 40°C. The reaction was initiated by the addition of $MgCl_2$ (11 mM). Aliquots were removed from the reactions at the indicated times, and the reactions were stopped by mixing the aliquots with equal volumes of 95% formamide in 25 mM EDTA. The wt precursor without nucleoside substrate (–); wt precursor with 0.2 mM G (G). Samples were analyzed as described for Fig. 2.

the binding of free G for the 5′ splice-site attack (*19*).

These data suggest that group I introns could use a nucleoside other than G in the splicing reaction. With the A:U variant (*13*), splicing-related activities were observed with the substrates r2AP and 2,6-diaminopurine ribonucleoside. In our report, another set of changes in the intron resulted in splicing activities with rP and A. Of these four substrates, the likely alternative to G, for splicing in vivo, is A. However, the variant splicing reaction with A requires high nucleoside concentrations and even then appears to proceed more slowly than the splicing of the wt precursor with G. It may be possible to introduce additional changes so that A will work in vivo. If so, it can be argued that either the specificity for G is arbitrary but difficult to change or is imposed for reasons not directly related to the splicing reaction. If, however, changes cannot improve A-dependent splicing, it may be that G works best and thus was selected on the basis of optimal splicing activity.

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14. The plasmid used in this study (pBFSN1) is a modified form of pBGST7, which contains the *Tetrahymena* group I intron sequence and short flanking exons cloned into the multicloning site of pUC18 and a T7 promoter sequence inserted into a Hae II site of the vector [M. D. Been and T. R. Cech, *Cell* **47**, 207 (1986)]. The plasmid pBFSN1 was generated by the insertion of an f1 origin of replication into the Nde I site of the vector, and two unique restriction sites in the intron sequence were generated by oligonucleotide-directed in vitro mutagenesis. For the mutagenesis, single-stranded U-containing DNA was prepared for use as a template [T. A. Kunkel, J. D. Roberts, R. A. Zakour, *Methods Enzymol.* **154**, 367 (1987); J. Vieira and J. Messing, *ibid.* **153**, 3 (1987)]. An Stu I site was added between P5b and P5a with a U162A change, and an Nsi I site was added to the end of P8 with a U289C change. As far as we could determine, these two changes had no effect on splicing activity, and this sequence was designated wt for this study. The G-binding site (*13*) was mutated by the use of two oligonucleotides designed to introduce random base changes at positions 264 and 311. All six individual base changes at positions 264 and 311 were isolated, and the three double mutations, which would restore a 264:311 Watson-Crick base pair, were constructed by the recloning of individual restriction endonuclease–generated fragments. Precursor RNA was made by in vitro transcription of Sca I– or Bam HI–digested plasmid DNA with T7 RNA polymerase. Sca I cuts within the intron sequence and results in a precursor that contains a 274-nucleotide 5′ exon and a truncated intron that ends five nucleotides short of the 3′ splice site; precursors made from Bam HI–cut plasmid DNA have a 5′ exon the same size, a full size intron (413 nucleotides), and a 265-nucleotide 3′ exon (the Bam HI site was introduced with the f1 origin sequence at the Nde I site).
15. Cleavage in the absence of nucleoside is due to hydrolysis at the 5′ splice site and by hydrolysis at the 3′ splice site followed by circularization to the 5′ splice site (*8*). For the C:G variant, we observed an enhanced rate of nucleoside-independent 5′ splice-site cleavage that was pH-dependent. This increased rate of hydrolysis at the 5′ splice site may also account for the production of ligated exons in the triple mutant (G264C:C311G:G414A) in the absence of added adenosine (Fig. 4).
16. A. T. Perrotta and M. D. Been, unpublished data.
17. The A (Sigma) and ATP (Pharmacia and U.S. Biochemicals, Cleveland, OH) were used as purchased without further purification; it is possible that some of the splicing activity observed with A and ATP resulted from small amounts of contaminating G or inosine.
18. The velocities (ν) of the reactions were determined [calculated as the fraction of Sca I–runoff (*14*) cleaved per minute during the initial phase of the reaction] under the same conditions as described for Fig. 4, but with varying nucleoside concentrations. The K_m and V_{max} were obtained from Eadie-Hofstee plots (ν versus $\nu/[S]$; $[S]$ is the nucleoside concentration). For the C:G variant, the V^{rP}_{max} (± SE) = 0.18 (±0.03) min^{-1}, K^{rP}_m = 0.3 (±0.05) mM, V^{A}_{max} = 0.17 (±0.05) min^{-1}, and K^{A}_m = 1.2 (±0.2) mM. For the wt sequence with G, V_{max} = 0.8 (±0.1) min^{-1} and K_m = 0.01 (±0.005) mM.
19. N. K. Tanner and T. R. Cech, *Biochemistry* **26**, 3330 (1987).

20. We thank C. Fierke, S. Rosenstein, and A. Kendall for comments on the manuscript; G. Vergara for photographic work; several members of the Boulder RNA group for comments on these results; and M. Hershfield for making a number of purine ribonucleoside compounds available to us. Supported by NIH grant GM-40689, an Institutional Award from the American Cancer Society (IN-158D), and a Junior Faculty Research Award (JFRA-233) from the American Cancer Society (M.D.B.).

12 October 1990; accepted 20 December 1990

Dynamic Tracking of Cardiac Vulnerability by Complex Demodulation of the T Wave

BRUCE D. NEARING, ALICE H. HUANG, RICHARD L. VERRIER*

A link is found between T wave alternans and vulnerability to ventricular fibrillation, and a new approach is provided for quantification of susceptibility to malignant arrhythmias. Complex demodulation reveals that alternation of the electrocardiogram is concentrated during the first half of the T wave, coinciding with the vulnerable period of the cardiac cycle. During myocardial ischemia and reperfusion, there are marked increases in the degree of T wave alternans that parallel the established time course of changes in vulnerability. The influence of the sympathetic nervous system in arrhythmogenesis is also accurately detected. Ultimately, complex demodulation of the electrocardiogram could provide a technique for identification and management of individuals at risk for sudden cardiac death.

SUDDEN CARDIAC DEATH, WHICH claims over 350,000 lives annually in the United States, results from abrupt disruption of heart rhythm, primarily in the form of ventricular fibrillation. Death is due not to extensive cardiac injury but rather to transient neural triggers that impinge on the electrically unstable heart (*1–3*). Identification of individuals at risk for sudden cardiac death remains a major objective in cardiology. Programmed cardiac electrical stimulation provides quantitative information but introduces the hazard of inadvertent induction of ventricular fibrillation. Quantification of T wave alternans is a promising approach because of its intrinsic safety and because of the consistent occurrence of T wave alternans before fibrillation under diverse conditions including coronary artery occlusion, hypothermia, Prinzmetal's vasospastic angina, and the long Q-T syndrome (*4–7*). Smith and co-workers (*4*) and Adam and co-workers (*8*) showed a correlation between fluctuations in overall energy of the T wave and the ventricular fibrillation threshold during coronary artery occlusion and hypothermia in dogs.

We studied a total of 16 adult mongrel dogs (20 to 30 kg) of either sex in accordance with the standards of the scientific community (*9, 10*). The animals were premedicated with morphine sulfate (2 mg per kilogram of body weight, subcutaneously) and anesthetized with α-chloralose (150 mg kg^{-1}, in-

Georgetown University School of Medicine, Washington, DC 20007.

*To whom correspondence should be addressed.