Title
Analyzing mechanisms of alternative pre-mRNA splicing using in vitro splicing assays.

Permalink
https://escholarship.org/uc/item/9nm9c7g7

Journal
Methods (San Diego, Calif.), 37(4)

ISSN
1046-2023

Authors
Hicks, Martin J
Lam, Bianca J
Hertel, Klemens J

Publication Date
2005-12-01

DOI
10.1016/j.ymeth.2005.07.012

Copyright Information
This work is made available under the terms of a Creative Commons Attribution License, available at https://creativecommons.org/licenses/by/4.0/

Peer reviewed
Analyzing mechanisms of alternative pre-mRNA splicing using in vitro splicing assays

Martin J. Hicks, Bianca J. Lam, Klemens J. Hertel *

Department of Microbiology and Molecular Genetics, College of Medicine, University of California, Irvine, CA 92697-4025, USA

Accepted 6 July 2005

Abstract

The development of in vitro assays to analyze pre-mRNA splicing resulted in the discovery of many fundamental features characterizing splicing signals and the machinery that completes this process. Because in vitro assays can be manipulated by various biochemical approaches, the versatility of investigating alternative pre-mRNA splicing in the test tube appears endless. Importantly, modifications in reaction conditions can lead to the accumulation, isolation, and characterization of reaction intermediates, a prerequisite for gaining mechanistic insights into how the spliceosome carries out intron removal, and how regulatory elements assist the general splicing machinery in defining splice sites and alternative exons. These considerable experimental advantages have made the in vitro splicing system a standard assay, even though this approach is independent from RNA transcription and other RNA processing events, and in some respects deviates from the natural process of mRNA biogenesis. Here, we describe the tools and techniques necessary to carry out in vitro splicing assays. Analyses of various experimental designs are presented to highlight the approaches taken to gain insights into the mechanisms by which splice site recognition and activation are communicated with the general splicing machinery. Methods to measure the kinetics of splicing, to observe the formation of the pre-spliceosomal complexes, and to manipulate and modify the in vitro system to resolve the regulatory influences in alternative splicing are presented.

1. Introduction

The analysis of adenovirus gene expression demonstrated that intron removal occurs post-transcriptionally at the pre-mRNA level [1–3]. Within a few years, it was shown that single gene transcripts resulted in various mRNA products, which later were revealed to be alternative spliced isoforms [4–6]. It was the advent of the in vitro splicing reaction using whole cell extract, and now exclusively nuclear extract, that eventually led to the description of many fundamental rules that define how exons and introns are recognized and to detailed molecular and structural insights into the machinery that completes this process [7]. Specific aspects of the splicing reaction were being unraveled using the in vitro splicing system. The characterization of the 3′–5′ phosphodiester bond at the splice junction [8], the formation of the intermediate lariat, and thus the identification of the branchpoint sequence necessary for the first catalytic step [9], and the role of U1 snRNP and other snRNPs in pre-mRNA splicing were mostly deduced through in vitro analysis [8,10–16]. Combining this information with the ability to separate pre-spliceosomal complexes using native gel electrophoresis gave rise to the ordered assembly pathway of the spliceosome [17–20]. Beyond the initial 5′ and 3′ splice site recognition signals, the characterization of splicing enhancer or silencer elements and their trans-acting factors effectively showed that it is not simply the splice site that activates splicing [21–24]. Many other studies have provided detailed descriptions of the composition [25], macromolecular structures [25–27], and rearrangements of spliceosomal complexes [28] and their roles in directing alternative splice site choice [29]. The advances made using in vitro splicing approaches are too numerous to list in its entirety. Suffice to say, the in vitro splicing assay has been and will be a valuable tool in deciphering the mechanism of splicing.
Although cell transfection experiments more closely resemble the natural context and process of pre-mRNA splicing, the in vitro system offers an experimental flexibility that allows investigators to design protocols directed towards specific aspects of the splicing reaction. The major benefit lies within the ability of biochemical manipulation, which, combined with use of minigene and heterologous pre-mRNA test substrates, enables to measure the influence of RNA elements and their trans-acting factors on the efficiencies and kinetics of regulated splice site selection. Considering the great variability that exist among 5' and 3' splice site recognition signals within higher eukaryotic genomes, these biochemical approaches will be crucial for the characterization of the many enhancer and silencer elements that have yet to be discovered. Lastly, the in vitro splicing assays are easily manageable. They do not require transfection and maintenance of cell lines, and results can be observed within hours.

However, even though the in vitro system has greatly facilitated our understanding of the splicing mechanism and its future contribution will be invaluable, the assay comes with limitations. At the outset, maximal rates of intron removal in vitro are slower than rates determined in vivo [30]. For example, the rate of β-globin intron 1 removal in vivo has been determined to be ~30 h⁻¹ [31]. The rate obtained for the identical intron removal event in vitro is at least one order of magnitude slower (1.2 h⁻¹, Fig. 1). At present, the origin of these differences are not known, in part because the rate-limiting step for the in vitro assay has not yet been determined. A reasonable explanation for this difference may be the lack of compartmentalization when using nuclear extracts. Thus, the difference in observed splicing efficiencies could simply be explained by concentration difference. In addition, in vitro transcription of pre-mRNA, its purification, and splicing reaction efficiencies restrict the size of the test substrate to be less than ~2000 base pairs. Thus, the in vitro splicing assay relies heavily on the study of short mini-genes that often are only a derivative of the endogenous gene. As many other processes take place in nuclear extracts, artifacts should be considered, and product size and character should be carefully analyzed. Finally, the analysis of splicing alone neglects the effects of other gene expression events, such as transcription, capping, and polyadenylation. However, the recent development of in vitro assays combining nuclear processes, such as the coupling of transcription and splicing [32] [M. J. H. and K. J. H., unpublished] [see also Natalizio and Garcia-Blanco in this volume], will allow measurements in a system presumably closer related to the in vivo condition.

2. Description of methods

2.1. Extract preparation

The in vitro study of alternative splicing is typically carried out in nuclear extracts prepared from HeLa cells. However, nuclear extracts from Drosophila embryos, such as Kc extract [33], Weri-1 retinoblastoma cells [34], and rat prostate AT3 cells [35] are also well documented. Since the efficiency of the in vitro splicing reaction is highly dependent on the quality of the extract used, care should be taken during its preparation. Detailed protocols for the preparation of nuclear extracts have been published in the past [36-39] or can be accessed through the internet (http://jeeves.mmg.uci.edu/hertel/protocols.html). We will therefore limit the discussion to general comments. The extract preparation will result in two major fractions, the nuclear extract and the cytoplasmic extract, also referred to as S100 extract. Whenever possible, batch preparations from relatively large volumes of suspension cells (50 L) should be used to generate sufficient amounts of extract (~60 mL of nuclear extract and ~40 mL of S100 extract) to last for the duration of a long-term project. Care should be taken not to exceed the swelling of the pelleted cells beyond the recommended 3× packed cell volume. Failure to do so will result in dilute and less active extract. Cytoplasmic extract is generated from the supernatant of pellet ed nuclei and the addition of a precipitation buffer that contains MgCl₂. The MgCl₂ treatment during the cytoplasmic extract preparation results in the specific precipitation of serine/arginine (SR)-rich proteins that are essential for pre-mRNA splicing. Therefore, cytoplasmic extract does not contain SR proteins and typically has dilute concentrations of factors required for constitutive splicing. However, because the activity of the S100 extract can be complemented by the addition of recombinant SR proteins, S100 extracts have been invaluable for evaluating SR protein function in pre-mRNA splicing [40,41]. Nuclear extract, on the other hand, contains all SR proteins required for pre-mRNA splicing and small nuclear ribonucleoprotein (snRNP) components of the splicing machinery. All extracts should be tested for specific activity using splicing substrates with known splicing efficiencies to normalize for variability between different extract preparations.

2.2. RNA preparation

RNAs used in splicing reactions are commonly made by run-off transcription using T3, SP6, or T7 bacteriophage RNA polymerases. In vitro transcription reactions contain 1× reaction buffer, 0.05 μg/μL linear DNA with a bacteriophage promoter, 2.5 μCi/μL [³²P-α]UTP, 0.4 mM ATP, 0.4 mM CTP, 0.1 mM UTP, 0.1 mM GTP, 2 mM m⁷G(5')ppp(5')G (cap analog), 2 mM DTT, 0.8 U/μL ribonuclease inhibitor, and 0.75 U/μL RNA polymerase. Reaction are incubated at 37 °C for 2 h, gel purified using denaturing PAGE, eluted from the gel (elution buffer: 0.5 mM NaAc, pH 5.6, 0.1% SDS, 10 mM Tris, pH 7.5, 1 mM EDTA), ethanol precipitated, and resuspended in dH₂O. The addition of the cap analog results in a protected 5’ end that extends the half-life of the test pre-mRNA when incubated in extracts. In many cases, the presence of a 5’ cap also increases the efficiency of the splicing reaction [42].
2.3. In vitro splicing reaction

A standard splicing reaction contains 300 cpm/μL RNA (0.01–0.1 nM), 30% HeLa nuclear extract or 40% HeLa S100 cytoplasmic extract, recombinant proteins if necessary, 1 mM ATP, 20 mM creatine phosphate, 3.2 mM MgCl\textsubscript{2}, 0.25 U/μL ribonuclease inhibitor, 1 mM DTT, 72.5 mM KCl, 12 mM Hepes–KOH, pH 7.9, and 3% PVA. The reaction volume is typically 25 μL, however, smaller volume reactions are equally efficient. Splicing reactions are incubated at 30°C for a minimum of 90 min. Following incubation, reactions are proteinase K digested in 200 μL with 10 mM Tris, pH 7.5, 1% SDS, 0.15 mM NaCl, 10 mM EDTA, 0.25 mg/mL glycerol, and 0.25 mg/mL proteinase K for 15 min at 37°C, phenol chloroform extracted, and ethanol precipitated prior to denaturing PAGE separation. Splicing reactions are then subjected to PhosphorImager analysis. Bands are detected on a BaFBr:Eu screen when radioactive emission causes localized phosphor oxidation. When the screen is illuminated by visible light (scanning), a reduction reaction occurs that causes release of photons. Photons are detected by a photomultiplier tube which produces a quantitative image. PhosphorImager analysis has a linear detection range over 5 orders of magnitude, while the linear range of film is limited to only 1.5 orders of magnitude.

2.4. Optimization of in vitro splicing reactions

Splicing of a particular transcript can be optimized in vitro so that it mimics in vivo splicing patterns. Extract concentrations from 10 to 50% are typically used to determine an optimal window for the analysis of splicing efficiencies in substrates with varying splice site, enhancer, and/or silencer strength. The potency of recombinant trans-acting factors, such as SR proteins, should also be tested with varying extract concentration. Polyvinyl alcohol (MW 30,000–70,000) is a high molecular weight polymer that concentrates macromolecules by volume exclusion and has been reported to enhance splicing, most likely by increasing the effective concentration of splicing components near pre-mRNA [42]. Optimal PVA concentration should be determined for each transcript by titrating from 0 to 3%. Another common method used to optimize splicing reactions is the substitution of KCl and MgCl\textsubscript{2} with KAc or KGlu and MgAc\textsubscript{2}, respectively. The use of acetate or glutamate instead of chloride as a counter ion allows splicing reactions to be performed at potassium concentration of 130 mM, which more closely mimics intracellular concentrations, instead of the optimal KCl concentration of 60 mM [42]. Indeed, it has been reported that the use of acetate or glutamate counter ions increases RNA stability, splicing intermediate products, and splicing products. It is hypothesized that chloride anions disrupt protein–nucleic acid interactions while acetate and glutamate have less disruptive effects [43].

2.5. Modification of in vitro splicing for characterization of RNA/protein and RNA/RNA interactions

2.5.1. Analysis of spliceosomal complexes using native gel electrophoresis

The in vitro splicing protocol described can be modified for the analysis of RNA/protein interactions. It is currently thought that the spliceosome assembles or rearranges in a stepwise manner onto pre-mRNA elements [44,45]. These complexes (H, E, A, B, and C) can be isolated through simple modification of the splicing protocol. E complex (splicing commitment complex consisting of U1 snRNP, U2AF, and loosely associated U2 snRNP) are enriched by carrying out a splicing reaction in the absence of ATP and creatine phosphate. Residual ATP in nuclear extracts is depleted by incubating extracts at 37°C for 30 min. Splicing reactions with substrates under these conditions are incubated for up to 40 min. E complex can be separated from H complex (pre-spliceosomal complex consisting of heterogeneous nuclear ribonucleoproteins, hnRNPs) through gel filtration or native gel electrophoresis using agarose gels [20,46]. A complex (stable association of U2 snRNP), B complex (incorporation of U4/U6.U5-tri-snRNP), and C complex (catalytically active spliceosome) formation are carried out under standard splicing conditions with short incubation times ranging from 1 to 40 min depending on the desired complexes. Complexes can be isolated by gel filtration [46] or by native gel electrophoresis after the addition of heparin to 0.5 μg/μL to prevent nonspecific protein/RNA associations [17,20,47]. Complexes can also be isolated through affinity chromatography using biotinylated substrates [48] or antibodies to splicing factors of interest.

2.5.2. Analysis of protein/RNA and RNA/RNA interactions using UV crosslinking

To identify specific proteins binding directly to an RNA substrate, UV crosslinking is commonly employed on uniformly or site-specifically labeled RNA [46]. RNA is incubated in splicing reactions as described for complex formation, placed on parafilm wrapped around a metal block on ice, crosslinked under a 254 nm UV light source, RNase digested, separated on SDS–PAGE, and analyzed by Western blotting [46]. Direct RNA/protein contact would result in crosslinking of radiolabeled RNA to protein. Western blotting confirms the identity of the labeled protein(s). The distance of sample from UV light and the duration of crosslinking should be determined for each substrate.

RNA/RNA interactions, such as snRNAs to pre-mRNA substrate, can be assayed through UV crosslinking with psoralen, which intercalates within nucleic acid helices and forms crosslinks to pyrimidines on opposite strands upon irradiation with 365 nm UV light. The procedure for setting up a psoralen UV crosslinking reaction is the same as the analysis for proteins, except for the wavelength of UV light used and the method for identification of RNAs crosslinked to substrate. Common methods used
to identify crosslinked species include RNase H mapping, primer extension, and Northern analysis [49].

3. Data analysis

3.1. Measuring the kinetics of the in vitro splicing reaction

The protocol for the in vitro splicing reaction calls for trace amounts of the test pre-mRNA while maintaining an excess of spliceosomal components throughout the reaction. At these conditions, the processing of the radiolabeled pre-mRNA can be described by pseudo-first order kinetics, thus enabling simplified data analysis. A typical splicing profile is illustrated in Fig. 1A. For historical reasons, the test substrate shown, a mini-gene derived from the β-globin gene, has become one of the gold standards for in vitro splicing.

As is evident from the splicing profile (Fig. 1A) and the graphical representation (Fig. 1B), measurable amounts of spliced product appear only after 25 min of incubation. Thus, in vitro splicing reactions are accompanied by an initial lag period, the length of which typically depends on the efficiency of intron removal. In general, pre-mRNA substrates that are poorly spliced show longer lag periods, while good substrates have shorter lag periods. It is unclear what accounts for the lag in the splicing reaction. Presumably, exogenously added pre-mRNAs are initially coated with hnRNP that are abundant in nuclear extracts, and the competing association and dissociations between hnRNP and components of the spliceosome require time. Thus, the association of splicing factors with pre-mRNA substrates varies with splice site strength, and this difference may account for the differences in the length of the observed lag [50].

After the lag period, mRNA formation follows the profile characteristic for first-order reactions. An initial linear phase is followed by a hyperbolic approach to the endpoint of the reaction (Figs. 1A and B). It is within these windows that reliable kinetic rate measurements can be obtained. However, prior to fitting the reaction profile to a mathematical description for first-order reactions, the data set needs to be normalized to the initial lag. The normalized reaction profile is then fit to the equation, the data set needs to be normalized to the initial profile to a mathematical description for first-order reactions. An initial lag may account for the differences in the length of the substrate varies with splice site strength, and this difference may account for the lag in the splicing reaction. Presumably, exogenously added pre-mRNAs are initially coated with hnRNP that are abundant in nuclear extracts, and the competing association and dissociations between hnRNP and components of the spliceosome require time. Thus, the association of splicing factors with pre-mRNA substrates varies with splice site strength, and this difference may account for the differences in the length of the observed lag [50].

Further insight into the in vitro reaction can be obtained by evaluating the degradation profile of the pre-mRNA substrate and mRNA product. As demonstrated in Figs. 1A and C, the pre-mRNA substrate is more vulnerable to degradation during the initial lag period, consistent with the suggestion that the naked substrate may be accessible to ribonucleases during the recruitment phase of splicing factors. During this lag period, the substrate disappears quickly at $k_1 = 4 \text{ h}^{-1}$, but once spliced products begin to accumulate the degradation rate rapidly decreases to about $k_2 = 0.3 \text{ h}^{-1}$.
indicating that the association of the splicing machinery with the pre-mRNA and the protein complexes associated with the mRNA after intron removal are stable and provide significant protection from ribonucleases. To obtain these rate constants, the data in Fig. 1C was fit to a biphasic decay description, \( A = C_1 \times (e^{-k_1 t}) + C_2 \times (e^{-k_2 t}) \), where \( A \) is the fraction degraded, \( C_1 \) and \( C_2 \) are the fraction of fast and slow degrading RNAs, \( k_1 \) and \( k_2 \) are the apparent rate constants for fast and slow degrading RNAs, and \( t \) is the time. In a typical splicing reaction, \( \sim 75\% \) of the input RNA is degraded during the lag phase of the reaction (\( C_1 = 0.75, C_2 = 0.25 \)).

3.2. Analysis of enhancer-dependent pre-mRNA splicing

The biochemical depletion or the addition of recombinant factors involved in pre-mRNA splicing constitutes a major experimental advantage of the in vitro splicing assay. These manipulations in reaction conditions allow the experimentalist to investigate the contribution of trans-acting factors in the regulation of intron removal of various test substrates. The activation of 5′ splice site usage by an exonic splicing enhancer (ESE) located upstream of the splice site is shown as a representative example (Fig. 2). The 5′ splice site of the pre-mRNA (CAG/guuggu) deviates from consensus (CAG/guaagu), and therefore, is considered less than optimal. The addition of recombinant SR proteins that bind specifically to the enhancer element upstream of the 5′ splice site will determine to what degree enhancer elements can activate the weak 5′ splice site. As illustrated in Fig. 2, the addition of recombinant SR proteins results in an increased production of mRNA throughout the time course of the reaction. On one hand, single time point comparisons of the fraction spliced for each reaction conditions demonstrates significant variability in the magnitude of the observed difference in splicing efficiency. For example, after 30 min, the reaction in the presence of recombinant SR protein appears to have proceeded \( \sim 5 \) times more efficient, while after 120 min the difference appears to be only \( \sim 2\)-fold. On the other hand, the determination of the observed rate of splicing takes into account all time points measured to calculate the difference in pro-

Fig. 2. Activation of enhancer-dependent splicing. (A) Diagram of the enhancer-dependent pre-mRNA and representative autoradiogram of the in vitro splicing profiles of this transcript over a 240-min time course. The reaction was performed in the absence (left panel) or presence (right panel) of saturating amounts of recombinant MS2-9G8 (250 nM) which binds specifically to the MS2-ESE located upstream of the 5′ splice site. Pre-mRNA transcript, spliced product, and intermediate lariat bands are indicated between the autoradiograms. The percent spliced for each time point is indicated below each lane. (B) Quantitation of the data in (A). Percent spliced is defined as (cpm product)/(cpm substrate + cpm product). The graph illustrates the fit of the data to a pseudo-first order rate description for product appearance, \( A = C \times (1 - e^{-kt}) \), after normalization to the lag (25 min). The rates determined are indicated on the right.
cessing efficiency, and this difference turns out to be 4-fold (Fig. 2B). This example demonstrates how kinetic analysis of reaction profiles more faithfully reflects the true difference between tested reaction conditions.

3.3. Inferring splicing mechanisms from in vitro splicing assays

As illustrated in Fig. 2, the comparison of different reaction conditions is a valuable approach to compare the relative strength of splice sites, enhancers, silencers, and the trans-acting factors involved in splicing a particular substrate. However, mechanistic details of the splicing reaction can also be inferred from rate analysis of in vitro splicing reactions. For example, to determine if a splicing enhancer complex activates one splice site or a pair of 5’ and 3′ splice sites, splicing rates were compared in substrates where enhancer-dependent splice sites were separated across an intron or an exon (Fig. 3A). If the 5’ and 3′ splice sites were recognized independently,

Fig. 3. Titration of MS2-RS in the presence and absence of Tra/Tra2. (A) Diagram of two-exon and three-exon minigenes. The two-exon substrate contains ESE-dependent 5’ and 3′ splice site on separate exons. The 5’ splice site is activated by the addition of Tra/Tra2. The 3′ splice site is activated by the addition of MS2-RS fusion proteins. The three-exon substrate contains ESE-dependent 5’ and 3′ splice sites within the internal exon. Splice sites are activated as described in the two-exon substrate. (B) MS2-RS titration in 25% HeLa nuclear extract with or without 400 nM Tra/Tra2. Identities of unspliced and spliced RNAs are indicated on the right. Splicing efficiency is indicated on the bottom. (C) Splicing efficiency was graphed as a function of MS2-RS concentration and then fitted to an isotherm. Dissociation constants are indicated in the graph.
i.e., in two different steps, their activation by ESEs would occur synergistically. Alternatively, if the 5′ and 3′ splice sites were recognized concurrently, their activation by ESEs would be additive. To enable independent activation of each splice site, the native enhancer from the fru female specific exon (fruRE) was used for the activation of the weak 5′ splice site and a MS2 hairpin ESE was used for the activation of the weak 3′ splice site (Fig. 3A). The activators specific to the fruRE (Transformer, Tra and Transformer 2, Tra2) and/or the MS2 enhancer (recombinant MS2-SR fusion proteins) were purified from over-expression systems and added to in vitro splicing reactions. In the first set of experiments, the efficiency of pre-mRNA splicing of the single intron substrate (Fig. 3A) was investigated. Rate constants were determined for each condition tested (no recombinant proteins added, Tra/Tra2, MS2-RS, and Tra/Tra2/MS2-RS) and normalized to the rate constant where no enhancer complexes formed. The activation of weak splice sites by ESEs located on different exons was found to occur synergistically (Table 1). These results suggest that the recognition of each suboptimal splice site on different exons constitute an independent step during spliceosome assembly.

However, synergy could have also occurred by the cooperative binding interactions between the two enhancer complexes. To test this possibility, the assembly efficiency of the MS2-RS enhancer complex was determined in the presence or absence of the Tra/Tra2-dependent enhancer complex. As expected, the overall efficiency of intron removal was more efficient in the presence of both enhancers, but the concentration of MS2-RS required for half-maximal activation remained nearly unchanged (Figs. 3B and C). This finding demonstrates that the synergistic activation of intron removal is not caused by advantageous binding interactions between the ESEs tested and that each enhancer complex assembled independently.

Next, the activation of weak splice sites across the same exon was examined. When the ESEs were situated within one exon that is flanked by suboptimal splice sites, the activities of both enhancers were observed to be additive (Table 1). These results demonstrate that an individual enhancer complex is sufficient to activate both weak splice sites of one exon. Therefore, ESEs recruit a complex that minimally contains all factors necessary for initial recognition of the 3′ and 5′ splice sites [51].

4. Summary

The use of the in vitro splicing assay has led to the discovery of many fundamental characteristics of the splicing reaction. Although the standard in vitro splicing assay limits the investigation to RNA splicing of minigenes uncoupled from transcription and other RNA processing events, the in vitro splicing assay has emerged as a powerful tool to investigate splicing mechanisms, chemistry, and structural rearrangements of the spliceosome. This is primarily due to the extensive flexibility of the in vitro assay, which allows the adjustment of reaction conditions, biochemical modification of extracts, and the isolation and characterization of reaction intermediates. In addition to these experimental benefits, kinetic analyses of the splicing reaction generate valuable insights into the mechanisms of the splicing reaction. Significantly, a comparison of reaction profiles provides a more accurate and compelling argument for any observed variability in splicing efficiency than the comparison of single time points.

The in vitro splicing assay has been and will continue to be a methodology commonly used in the quest to decipher the rules that dictate splice site recognition and pairing, to describe the rearrangements during spliceosomal assembly, and to elucidate the dynamic interactions within the catalytic center, especially as one of the goals is to eventually obtain a three-dimensional representation of the spliceosome “caught in the act” of intron excision. The challenge that lies ahead is to continue to make evident that the mechanistic insights gained from the in vitro assay, in fact, are relevant in the course of the splicing reaction as it takes place in the cell.

Acknowledgments

We thank the Hertel laboratory for helpful comments on the manuscript. This work was supported by a NIH training grant GM 07311 (B.J.L.) and a NIH Grant GM 62287 (K.J.H.).

References