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Antisera to Poly(A)·Poly(U)·Poly(I) Contain Antibody Subpopulations Specific for Different Aspects of the Triple Helix†

Lynne C. Rainen* and B. David Stollar

ABSTRACT: Rabbit antibodies to the triple-helical polynucleotide poly(A)·poly(U)·poly(I) were fractionated into three major antibody populations, each recognizing a different conformational feature of the triple-helical immunogen. Two distinct populations were purified from precipitates made with poly(A)·poly(U)·poly(U) and poly(A)·poly(I)·poly(I). The former reacted with double-stranded poly(A)·poly(U) or poly(I)·poly(C), and similar populations could be purified with either double-stranded form. The second population recognized the poly(A)·poly(I) region of the triple helix, and the third required all three strands for reactivity. These immunochemical studies suggest that the poly(A) and poly(U) have the same orientation in the triple-helical poly(A)·poly(U)·poly(I) as in the double-helical poly(A)·poly(U), in which they have Watson-Crick base pairing.

Several antibodies to nucleic acids have been shown to be specific for conformational features of helical structures. Some can react specifically with double-stranded RNA (Nahon et al., 1967; Schwartz and Stollar, 1969; Stollar, 1970; Plescia et al., 1969; Talal et al., 1971) or RNA-DNA hybrids (Stollar, 1970) and can identify corresponding structures in samples of biological origin (Silverstein and Schur, 1970; Stollar and Stollar, 1970). Anti-triple-helix antibodies can differentiate triple-stranded structures built on poly(A) from those built by poly(dA) (Stollar and Raso, 1974). It has been suggested that triple-helical nucleic acids may be involved in viral replication (Bishop et al., 1965), gene regulation (Miller and Sobell, 1966; Britten and Davidson, 1969), bacterial transcription (Zubay, 1958, 1962), and chromosome structure (Crick, 1971), but the triple-helical forms have not been clearly demonstrable in vivo; therefore, antibodies that recognize such structures could be useful reagents to test their presence in naturally occurring nucleic acids. Additionally, anti-triple helix antibodies are important in defining conformation-dependent antigenic determinants of nucleic acids and in providing a model for specific protein–nucleic acid interactions.

De Clercq et al. (1975) have used a sensitive assay based upon interferon induction to demonstrate the formation of the triple helix poly(A)·poly(U)·poly(I) in displacement reactions involving polynucleotides. They have further demonstrated the existence of this polymer by physical-chemical techniques. We report here that antibodies induced by the triple-stranded polyribonucleotide poly(A)·poly(U)·poly(I) can be separated into subpopulations that are specific for three different aspects of the helix, and that these antibodies may be of help in the characterization of the polymer's structure.

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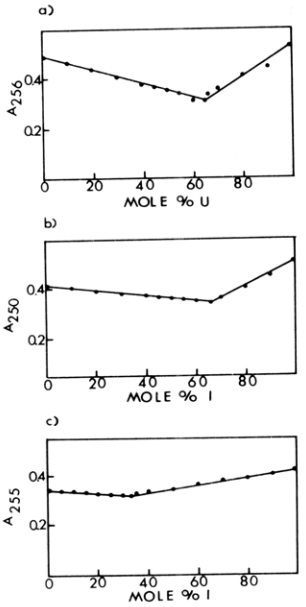

FIGURE 1: Mixing curves of triple helices. Homopolymers in PBS were mixed in varying proportions and incubated at 25 °C for 1 day and at 4 °C for 4 days. Total nucleotide concentration was held constant at 1 μmol/mL, and samples were diluted 20-fold for spectrophotometric readings. (a) Poly(U) added to poly(A); (b) poly(I) added to poly(A); (c) poly(I) added to poly(A)·poly(U).

Materials and Methods

Synthetic polynucleotides were purchased from P-L Labs, Inc. Stock solutions were prepared at a concentration of 1 μmol/mL of nucleotide in PBS[1] (0.15 M NaCl–0.01 M sodium phosphate, pH 7.2). Concentrations were determined spectrophotometrically from the molar extinction coefficients: $\epsilon_{260} = 9.2 \times 10^3$ for poly(A); $\epsilon_{260} = 9.7 \times 10^3$ for poly(U); and $\epsilon_{248} = 10.2 \times 10^3$ for poly(I).

Formation of Double- and Triple-Stranded Polymers. Mixtures of varying proportions of the stock solutions of homopolymers were prepared in acid-washed tubes; the total nucleotide concentration was held constant at 1 μmol/mL. The polymer mixtures were allowed to anneal for 1 day at 25 °C and 4 days at 4 °C. Samples were diluted 20-fold for spectrophotometric readings.

Immunization. Three female New Zealand white rabbits were first immunized at multiple intradermal sites with 100 μg of poly(A)·poly(U)·poly(I) complexed with an equal weight of methylated bovine serum albumin (Sigma Chemical Co.) and emulsified in complete Freund's adjuvant. Similar injections, but with incomplete Freund's adjuvant, were given on days 7 and 14, followed by an intravenous boost of complexes without adjuvant on day 21. Sera were obtained on day 27. For later sera, the rabbits were given intravenous booster injections 6 days before bleeding. Sera were heated at 56 °C for 30 min before use.

Immunoprecipitation. For quantitative preciptin assays, 0.1 mL of serum, 2 to 40 μg of antigen, and PBS were incubated in a final volume of 0.3 mL at 37 °C for 2 h and 4 °C overnight. Precipitates were centrifuged, washed three times with 1-mL portions of cold PBS, and redissolved in 0.1 N NaOH. Protein concentration was determined by the method of Lowry et al. (1951).

[1] Abbreviations used: PBS, 0.15 M NaCl–0.01 M sodium phosphate, pH 7.2; poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); poly(I), poly(inosinic acid); DEAE, diethylaminoethyl.

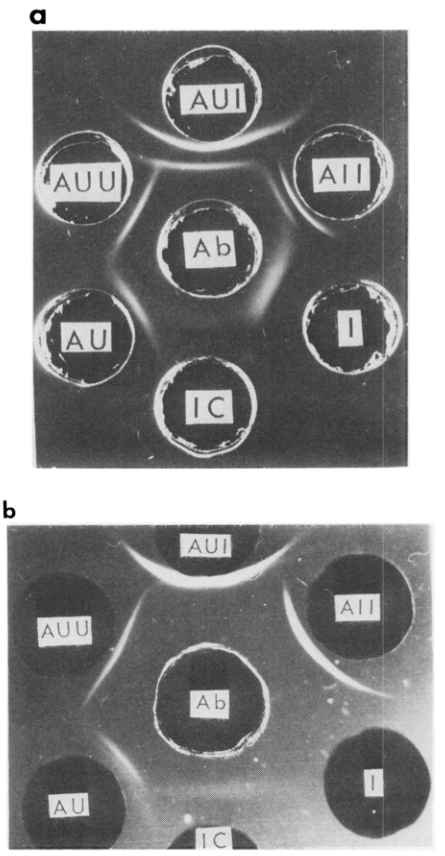

FIGURE 2: Immunodiffusion with: (a) whole serum 367b and (b) serum 367b absorbed with poly(I). For absorption, antibodies were precipitated by addition of 100 μg of poly(I) to 2.0 mL of serum; the resulting supernatant was passed through DEAE-cellulose in 0.025 M phosphate to remove excess poly(I). The gel was 0.8% purified agarose in PBS. Each antigen well contained 50 nmol of polynucleotide in 50 μL of PBS.

Antibody Purification. One to ten milliliters of serum was incubated with an equivalent amount of polymer, as determined from quantitative precipitin curves, for 2 h at 37 °C and overnight at 4 °C. Resulting precipitates were washed three times in cold PBS and resuspended in 2 mL of distilled water. The suspension was incubated at 50 °C for 10 min to denature the polymer and dissociate it from the antibody. Pancreatic RNase (80 μg) was added; the mixture was incubated for 2 h at 52 °C and was then brought to 0.15 M NaCl to solubilize the antibody globulin. The purified antibody population was then obtained by passing the digest through a Sephadex G-200 column with PBS as the running buffer. The 7S peak was pooled and stored frozen in PBS.

Absorbed Sera. Serum that had previously been precipitated with antigen as described above was dialyzed against 0.025 M sodium phosphate and passed through a DEAE-cellulose column equilibrated and developed with the same buffer, to remove any excess polymer.

Quantitative microcomplement fixation was performed as described elsewhere (Stollar, 1977).

Results and Discussion

Mixing Curves. It has previously been shown that the formation of the triple helix can be monitored by measuring the hypochromicity at a particular wavelength as a function of varying molar ratios of polymer (Stevens and Felsenfeld, 1964; DeClerq et al., 1975). In order to ensure that the immunogen poly(A)·poly(U)·poly(I) and the triple-stranded test antigens poly(A)·poly(U)·poly(U) and poly(A)·poly(I)·poly(I) were

TABLE I: Antibody Titers of Whole 367b Serum with Triple-Helical Polynucleotide Antigens.

Antigen	50% Max C F[a] Titer	μg of Ab ppt/mL	μg of Ab/50% C F[a] Titer
Poly(A)·poly(U)·poly(I)	28 000	1670	0.06
Poly(A)·poly(I)·poly(I)	11 500	1100	0.09
Poly(A)·poly(U)·poly(U)	4 300	660	0.15

[a] C F, complement fixation.

completely in the three-strand form, mixing curves were obtained for all three polymers (Figure 1). The mixing curves indicated that maximum hypochromicity was reached when polymer ratios were those favoring the triple-stranded conformation. Storage for long periods of time (3–4 months) or freezing and thawing several times had little or no effect on the serological reactivity or spectral properties of the triplexes.

Reactions with Whole Serum. All three rabbits responded to immunization with poly(A)·poly(U)·poly(I). A second-course serum, designated 367b, was characterized in particular detail. In immunodiffusion studies with whole serum, two lines of precipitation were seen with both the immunogen poly(A)·poly(I)·poly(I), indicating that the antigens as well as the antibody populations were heterogenous. In both cases, there were strong precipitin lines of concave curvature toward the antigen well and inner lines that fused with each other and appeared related to a line formed with poly(I) alone (Figure 2a). The picture was clarified when the serum was absorbed with poly(I). The poly(A)·poly(I)·poly(I) and poly(A)·poly(U)·poly(I) then each gave a single line of precipitation that was concave toward and close to the antigen well while the inner lines no longer appeared (Figure 2b). Thus the original inner line was due to excess poly(I) in the antigens. Additionally, the remaining precipitin band that formed with the immunogen spurred over that of poly(A)·poly(I)·poly(I). The homologous antigen also spurred over a precipitin line of partial identity given by poly(A)·poly(U)·poly(U). The latter line fused with one formed by the double-stranded poly(A)·poly(U) or poly(I)·poly(C). This suggested that some aspect of the triple helix is structurally similar to the double-helical form, a possibility that was explored further with purified antibodies as described below.

Reactions with all of the antigens mentioned above also occurred in quantitative precipitation (Figure 3). The strongest reaction occurred with poly(A)·poly(U)·poly(I). The order of reactivity of the other polymers was poly(A)·poly(I)·poly(I) > poly(A)·poly(U) = poly(A)·poly(U)·poly(U) (not shown) = poly(I)·poly(C) > poly(I) (not shown).

A similar ranking of reactivity occurred in quantitative complement fixation assays (Table I). The serum titer, defined as the reciprocal of the dilution required to give a curve with a peak of 50% complement fixation, was highest with poly(A)·poly(U)·poly(I), and the others reacted in the order: poly(A)·poly(I)·poly(I) > poly(A)·poly(U)·poly(U) > poly(A)·poly(U) (data not shown) > poly(I)·poly(C) (data not shown). The ratio of μg/mL of precipitable antibody to complement fixation titer for homologous antigen was 0.06, close to the mean value noted for several other homologous systems (Gruenewald and Stollar, 1973). With cross-reactive antigens, the ratios were 0.09 for poly(A)·poly(U)·poly(U) and 0.15 for poly(A)·poly(I)·poly(I). Thus the complement fixation reactivity was most efficient with the homologous reactant, and, while the complement fixation titer may provide an approximation of precipitable antibody for homologous antigen, it would markedly underestimate the value for cross-reactive antigens.

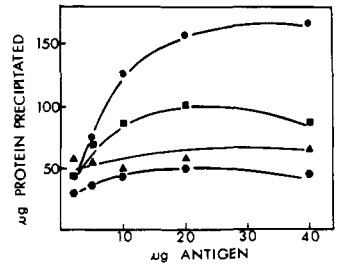

FIGURE 3: Immunoprecipitation of whole 367b serum with polynucleotide antigens. Two to forty micrograms of antigen, PBS, and 0.1 mL of whole serum were incubated in a final volume of 0.3 mL at 37 °C for 2 h and at 4 °C overnight. Precipitates were washed with PBS and redissolved in 0.1 N NaOH. Protein concentration was determined by the method of Lowry et al. (1951). Antigens: (●) poly(A)·poly(U)·poly(I); (■) poly(A)·poly(I)·poly(I); (▲) poly(A)·poly(U); (●) poly(I)·poly(C).

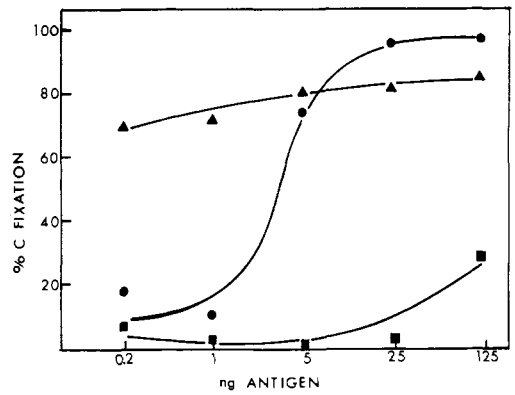

FIGURE 4: Quantitative micro-complement fixation of 367Pu(AII) with: (●) poly(A)·poly(U)·poly(I) ([Ab] = 0.375 μg/mL); (▲) poly(A)·poly(I)·poly(I) ([Ab] = 0.375 μg/mL); (■) poly(A)·poly(U)·poly(U) (1.5 μg/mL Ab).

Reactions of Purified Antibodies and Absorbed Serum. The reactions of whole serum with various polymers suggested the presence of distinct subpopulations of antibodies that were specific for varying portions of the triple helix. To test this hypothesis, the antibodies were fractionated by absorption and immunospecific purification. Purified antibodies were designated 367Pu followed by the polymer with which they were precipitated for the purification. Four purified populations were prepared: 367Pu(AUU); 367Pu(AII); 367Pu(AU); and 367Pu(IC). The residual serum after absorption with both triple helical cross-reactants was 367Abs(AUU,AII). The 367Pu(AII) population reacted well with the purifying polymer but very little with poly(A)·poly(U)·poly(U) (Figure 4). Reciprocally the 367Pu(AUU) reacted with very little poly(A)·poly(I)·poly(I) (Table II). Thus, these two distinct subpopulations recognized different structures. Interestingly, both populations reacted more effectively with poly(A)·poly(U)·poly(I), the original immunogen, than with the polymers used for their purification (Table II). Thus the poly(A)·poly(U)·poly(U) and the poly(A)·poly(I)·poly(I) each presented a different feature that resembled but was not completely identical with a part of the immunogen. The reactivity of each antibody population was apparently sufficient for precipitation when two of the three strands were recognized, but was further enhanced by the presence of the third strand.

TABLE II: Complement Fixation Titers of Fractionated Subpopulations of Anti-poly(A)·poly(U)·poly(I) Serum.

	Poly(A)·poly(U)·poly(I)	Poly(A)·poly(I)·poly(I)	Poly(A)·poly(U)·poly(U)
367Pu(AII)	220	58	*a*
367Pu(AUU)	240	*b*	145
367Abs(AUU,AII)	5200	*c*	*c*

[a] Zero C fixation at a 1:20 dilution. [b] Twenty percent C fixation at a 1:20 dilution. [c] Zero C fixation at 1:100 dilution.

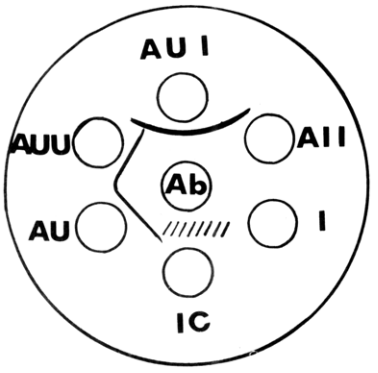

FIGURE 5: 367Pu(AU) immunodiffusion. The center well contained 60 μg of protein in 50 μL. Each antigen well contained 50 nmol of polynucleotide in 50 μL of PBS.

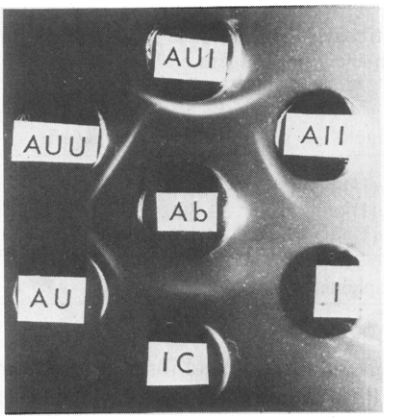

FIGURE 6: 367Pu(IC) immunodiffusion. The center well contained 180 μg of protein in 50 μL. Each antigen well contained 50 μmol of polynucleotide in 50 μL of PBS.

The serological distinctions between triple helices made of poly(A) + poly(U) or poly(A) + poly(I) were confirmed with antisera that were homologous for either one of them. Antiserum induced by poly(A)·poly(U)·poly(U) gave some cross-reactivity with poly(A)·poly(U)·poly(I) but none with poly(A)·poly(I)·poly(I). A reciprocal graded specificity pattern was obtained with antiserum induced by poly(A)·poly(I)·poly(I).

The above reactions of whole serum and purified fractions of serum 367b suggested that some antibodies reacted with both a double-stranded helix and a structurally similar aspect of the triple helix. This interpretation was strengthened when a population of antibodies specific for the double-stranded polymer poly(A)·poly(U) was purified from whole serum. With this 367Pu(AU), reactions of identity were seen in immunodiffusion with poly(A)·poly(U) and poly(A)·poly(U)·poly(U). Furthermore, this purified antibody population gave a single line of precipitation with poly(A)·poly(U)·poly(I), and the line was markedly concave toward the antigen well, as was the major line seen with whole serum. It spurred over the poly(A)·poly(U)·poly(U) line in a reaction of partial identity (Figure 5). The same pattern was seen with an antibody population purified with poly(I)·poly(C), a purely double-stranded polymer. The fact that the original immunogen gave the strongest reaction in both cases provided evidence that the purified antibody was in fact induced by a part of the triple helix and not by some free poly(A)·poly(U) in the immunogen. Indeed, antibody induced by poly(A)·poly(U) itself (Schwartz and Stollar, 1969) did react with the poly(A)·poly(U)·poly(I) in complement fixation; in this case the complement fixation titer was similar for both antigens, but threefold higher concentrations of the triple helix were required for a given level of reactivity. Whereas the homologous anti-poly(A)·poly(U) and the 367 Pu(Au) or 367Pu(IC) all recognized mainly the features presented by the poly(A) + poly(U) backbone arrangement, the third strand, poly(I), interfered with the binding of the former and enhanced the binding of the latter two.

In addition to reacting with poly(A)·poly(U) and (presumably) the double-stranded region of poly(A)·poly(U)·poly(U), the 367PuIC seemed to recognize a double-stranded region of poly(A)·poly(I)·poly(I) since there was no cross-reaction with the single-stranded poly(I) alone (Figure 6). This reactivity with poly(A)·poly(I)·poly(I) was not seen with 367PuAU.

Since poly(A) + poly(U) helices differ markedly from poly(A) + poly(I) helices and since antibodies that recognized the poly(A)·poly(U) double helix also recognized the poly(A)·poly(U)·poly(I), it appears likely that it is the poly(A) and poly(U) that form the Watson–Crick base pairing in the triplex and that poly(I) is the third strand in the major groove of the helix formed by the other two.

The absorbed serum, 367Abs(AUU,AII), contained antibody that was specific for a structure requiring all three strands of the immunogen, showing no complement (C) fixation reactivity with poly(A)·poly(I)·poly(I) or poly(A)·poly(U)·poly(U) (Table II). It also showed no reactivity in immunodiffusion with poly(A)·poly(U)·poly(U), poly(A)·poly(I)·poly(I), poly(A)·poly(U), or poly(I). Effective antigen was formed when poly(I) was added to poly(A)·poly(U). When the polymers were mixed in continuously varying proportions, the maximal amount of reactive antigen as judged by the lateral shift of the C fixation curve was present when the ratio of the three polymers was 1:1:1, i.e., when the poly(I) was about one-third of the total (Figure 7). This further indicates that antibody remaining in the serum after double-strand-specific antibodies were removed recognized only the triple-helical immunogen.

Conclusion

From our studies with poly(A)·poly(U)·poly(I) as an immunogen, we have found several populations of antibodies in whole serum. One recognized free excess poly(I) that must have been present in the immunogen; it was removed from serum selectively by absorption. Another recognized the poly(A)·poly(U) portion of the triple helix; a third one recognized the poly(A)·poly(I) portion, and a fourth population was specific for all three strands. With an immunogen as large as a triple-helical polynucleotide, subpopulations which are

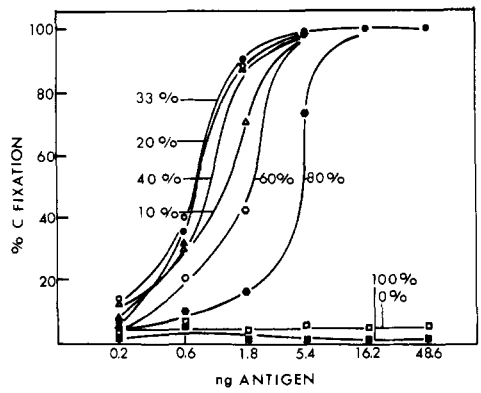

FIGURE 7: Quantitative micro-complement fixation of 367Abs-(AUU,AII) with poly(A)·poly(U) + continuously varying proportions of poly(I) as a percentage of total polynucleotide: (□) 0%; (△) 10%; (○) 20%, (●) 33%; (▲) 40%; (○) 60%; (●) 80%; (□) 100%.

specific for different aspects of the same molecule are not unexpected, the precedent having been seen in simpler antibody-hapten systems (Kreiter and Pressman, 1964). In their studies of a large "two-headed" hapten, one fragment of the hapten purified an antibody population that was specific for that portion but still had a higher affinity for the whole hapten than for the fragment alone. As in our population purified by poly(A)·poly(U), the antibodies had a binding site that could accommodate the portion used for purification plus additional neighboring features of the immunogen.

In evaluating the extent of an antigenic determinant on the triple helix, an important consideration must also be the dimensional limitations of the antibody combining site. An Fab fragment of a myeloma protein has a binding surface of about 25 × 20 Å (Amzel et al., 1974). The effective diameter of the poly(A)·poly(U)·poly(U) and the poly(A)·poly(I)·poly(I) triplexes have been determined to be 24.6 and 23.7 Å, respectively (Arnott and Bond, 1973a,b). This is a range compatible with the size of an antibody combining site which sees portions of all three strands, reaching across either the major or minor groove. It is also reasonable that a significant part of the antibody population should recognize mainly a smaller portion of the whole structure, such as a region encompassing two of the three strands.

While two of the three antibody populations do seem to react mainly with double-stranded portions of the triple strand, it is clear that the presence of the third strand still contributes to antibody recognition. This is illustrated by the fact that in C fixation both purified antibody populations 367Pu(AUU) and 367Pu(AII) prefer the immunogen to the purifying antigen. The reasons for this may be twofold: (1) antibody combining sites may involve largely two strands and part of the third, or (2) the presence of the third strand in the immunogen may subtly affect the backbone conformation of the other two strands such that the antibody combines more effectively with them when they are present in the three-stranded form.

Finally, although the structures of poly(A)·poly(U)·poly(U) and poly(A)·poly(I)·poly(I) have been determined by x-ray diffraction (Arnott and Bond, 1973a,b), the exact structure of the triple helix poly(A)·poly(U)·poly(I) is not well understood. It is not known whether poly(A) pairs with poly(U) in the familiar Watson-Crick configuration allowing the poly(I) to fit in the major groove of the double helix or whether the opposite arrangement is the case. The serological reactions we observed suggest that poly(A) and poly(U) have the same orientation in the triple-helical poly(A)·poly(U)·poly(I) as in the double-helical poly(A)·poly(U), in which they have Watson-Crick base pairing. Further studies, such as measurements of melting profiles of the triplex in the presence of various specific antibodies, may further help to elucidate the conformational relationship of the three strands.

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