# Effects of Antibody Binding upon Thermal Transitions of Polynucleotides†

Vic Raso* and Ronnye Schreiber

ABSTRACT: Antibodies directed against the double- or triple-helical features of poly(A)·poly(U) and poly(A)·poly(U)·poly(U) were elicited in rabbits. These different types of antibodies were purified by precipitation with the appropriate polynucleotide complex and monovalent Fab fragments were then prepared by papain digestion. Thermal hyperchromicity studies were performed to assess the influence of specific Fab binding upon the temperature-dependent separation of both double- and triple-helical structures into their constituent strands. Anti-$AU_{Fab}$ caused two concentration-dependent alterations in the melting profile of the AU → A + U transition. As increasing amounts of Fab were added, the extent of melting was progressively reduced and the melting temperature of the fraction which did undergo transition was concomitantly raised. Both effects were specific since heating in the presence of Fab derived from nonimmunized rabbits produced a hyperchromicity profile indistinguishable from that of poly(A)·poly(U) alone. In a kinetic study anti-$AU_{Fab}$ was found to influence both the rate and extent of base pair formation. Triple stranded poly(A)·poly(U)·poly(U) can dissociate in either one or two steps depending upon the $Na^+$ concentration (AUU (t1) → A + U + U, 0.15 M $Na^+$ or AUU (t2) → AU + U (t3) → A + U + U, 0.031 M $Na^+$). Direct separation into three random coils is retarded by anti-$AUU_{Fab}$ as evidenced by a substantial increase in t1. Anti-$AU_{Fab}$ and nonspecific Fab do not afford such stabilization. When anti-$AUU_{Fab}$ is present during the biphasic reaction, melting proceeds as one continuous transition instead of two discrete increments. Dependent upon Fab concentration, this modified dissociation can occur at a temperature which is 10 °C greater than t3. Nonspecific Fab does not alter this biphasic melting profile but anti-$AU_{Fab}$ has two effects. In 0.031 M $Na^+$ addition of this protein to poly(A)·poly(U)·poly(U) appears to promote a rapid melting of the third strand at 22 °C, a temperature at which the triple helix otherwise remains intact. When heating commences, this anti-$AU_{Fab}$ stabilizes the remaining double helix and t3 occurs at an elevated temperature. These results have been employed to elucidate the nature of the interaction between antibodies and double- or triple-stranded polynucleotides.

The specificity characteristics observed in nucleic acid immunochemistry can be divided into three main categories depending upon which features of these complex molecules are recognized by antibody (for reviews, see Levine & Van Vunakis, 1966; Plesica & Braun, 1967; Levine & Stollar, 1968; Goldfarb, 1968; Stollar, 1973; Locour et al., 1973; Stollar, 1975). Thus, there has been extensive study of antibodies which are predominantly directed against the chemical aspects of individual bases whether they be free or incorporated into a nucleotide or polynucleotide structure (Butler et al., 1962; Plescia et al., 1968; Erlanger & Beiser, 1964). Alternatively, antibodies may selectively interact with the ribose–phosphate backbone of relatively unstructured single-stranded polynucleotides. Reactivity is not dependent upon the conformation of the nucleic acid as long as these determinants are not sterically occluded (Barbu & Panijel, 1960; Panijel et al., 1966). In this respect the interaction is similar to that described for base-specific antibodies.

A third and totally different specificity has come to light through recent work which has resulted in the induction of antibodies directed toward the stereochemical configuration of polynucleotide complexes prossessing ordered secondary structure. Specificity studies have shown that antibodies elicited by double- and triple-stranded nucleic acids react with determinants dependent upon the interaction between the component strands (Nahon et al., 1967; Schwartz & Stollar, 1970; Stollar & Raso, 1974). They are not present on the individual polynucleotide chains in random configuration. While it is apparent that these antibodies interact with conformational features of the helix, neither the locus of binding nor the forces behind this antibody–nucleic acid reaction have been adequately elucidated.

This study examines the constraints which these antibodies exert upon thermal strand separations of double- and triple-helical complexes of poly(A)[1] and poly(U). With this information one may better define the contribution of each strand to the structural determinants bound by the two different antibody types. Helix formation is also allowed to proceed in the presence of these unique binding proteins to gain further insight into how they influence the elementary steps which comprise strand association–dissociation mechanisms. In order to pursue the nature of this interaction, bivalent antibodies have been enzymatically cleaved to produce monovalent Fab fragments. These retain antigen binding activity but, having only one site per molecule, do not possess the capacity to cross-link and precipitate polynucleotide antigens. Thus, any specific effects observed upon mixing these two reactants can be as-

† From the Division of Tumor Immunology, Sidney Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115. *Received May 12, 1977.* This investigation was supported in part by Grant CA 19589 from the National Institutes of Health. Part of this work was presented at the FASEB meetings (Raso, V. (1975) *Fed. Proc. Fed. Am. Soc. Exp. Biol. 34*, 976).

[1] Abbreviations used: poly(A)·poly(U), double-helical complex formed from equimolar amounts of poly(adenylic acid) and poly(uridylic acid); poly(I)·poly(C), double helical complex formed from equimolar amounts of poly(inosinic acid) and poly(cytidylic acid); poly(A)·poly(U)·poly(U), triple-helical complex formed from 1 mol of poly(adenylic acid) plus 2 mol of poly(uridylic acid); anti-AU, antibodies elicited by the double-helical determinants of poly(A)·poly(U); anti-AUU, antibodies elicited by the triple-helical determinants of poly(A)·poly(U)·poly(U); anti-$AU_{Fab}$ and anti-$AUU_{Fab}$, monovalent antigen binding fragments obtained by limited enzymatic digestion of the anti-AU or anti-AUU antibodies; Ra73-1, Ra73-2, and Ra73-3, designate the particular rabbit from which these antibodies were obtained.

0006-2960/78/0417-0968$01.00/0 

signed to the primary binding interaction or a change stemming directly from this interaction.

## Experimental Section

### *Materials*

Homopolymers poly(A), poly(U), poly(I), and poly(C) were purchased from Miles Laboratories, Inc. (Elkhart, Ind.). RNase, free of aggregates, and papain were obtained from Worthington Biochemical Corp. (Freehold, N.J.). Sephadex G-200 was from Pharmacia (Piscataway, N.J.). Methylated bovine serum albumin was from Sigma Chemical Co. (St. Louis, Mo.).

### *Methods*

*Preparation of Polynucleotide Complexes.* A stock buffer 1 M in cation concentration was made using NaOH which was adjusted to pH 7.2 with phosphoric acid before being brought to final volume. Polynucleotide complexes were formed by mixing appropriate molar proportions of each homopolymer so that the final concentration of the complex was $2 \times 10^{-3}$ M in 0.1 M $Na^+$ buffer. These were then heated at 100 °C for 10 min and allowed to cool slowly to room temperature. All reaction mixtures were brought to the stated $Na^+$ concentration with the stock buffer and contained in addition $3 \times 10^{-4}$ M ethylenediaminetetraacetic acid.

*Immunization Procedures.* Antibodies were induced in rabbits by injection of either poly(A)·poly(U) or poly(A)·poly(U)·poly(U) complexed to methylated bovine serum albumin and emulsified in complete Freund's adjuvant (Schwartz & Stollar, 1969; Stollar & Stollar, 1970).

*Antibody Isolation.* Serum from multiple weekly bleedings of individual rabbits was pooled and antibodies were isolated according to the method described by Stollar & Stollar (1970). This procedure involved specific precipitation of antibodies with the appropriate double- or triple-stranded polynucleotide followed by digestion of the washed precipitate employing ribonuclease at 52 °C in distilled $H_2O$, for 2 h. Gel filtration of the mixture on Sephadex G-200 provided separation of antibody from mononucleotides and ribonuclease. The antibody usually appeared in two peaks which were identified as γG and γM by heavy chain specific antiserum reagents. In all experiments reported here, only the pure 7S, γG antibody was used.

Each individual serum pool was tested to determine the proper amounts of polynucleotide needed to achieve maximum removal of antibody. Typically, 100 mg of purified antibody could be obtained from 50 mL of immune serum in a single isolation.

*Preparation of Fab Fragments.* Isolated anti-AU and anti-AUU antibodies were treated with papain and the mixture was chromatographed on carboxymethylcellulose to obtain purified Fab I fragments having a molecular weight of approximately 50 000 (Porter, 1959; Putnam et al., 1962). Antibody solutions were concentrated employing a diaflo ultrafiltration apparatus.

*Hyperchromicity Measurements.* Melting temperature experiments were performed at various $Na^+$ ion concentrations, in quartz cuvettes sealed with Teflon stoppers. A Gilford spectrophotometer with dual thermospacer set and a Hankee constant temperature circulator were used. Absorbance measurements at 260 nm were taken at 3 °C intervals allowing 15 min to attain equilibrium. Polynucleotide complexes were used at $2 \times 10^{-5}$ M (based upon nucleotide pairs or triplets), while Fab concentration was varied for different experiments. In a typical experimental set-up buffer served as a blank while polynucleotide alone, polynucleotide plus specific Fab and polynucleotide plus nonspecific Fab were run simultaneously. Before heating, the combined absorption at 260 nm of Fab plus polynucleotide was checked to determine if it deviated from a strictly cumulative value. These basal readings were stable for 15 min at room temperature, unless otherwise stated, and results were represented by a plot of the increase in the absorption at 260 nm vs. temperature. At exceedingly high temperatures, Fab plus polynucleotide produced anomalously high absorption, limiting the temperature range of study. While in many cases it is desirable to examine polynucleotide transitions at alternative wavelengths (Blake & Fresco, 1966), this was not done since the minimal absorption changes coupled with the high contributions from protein would yield unreliable data.

*Rate Determination.* Kinetic experiments for double-helix formation were performed at 26.5 °C using a Gilford spectrophotometer equipped with a recorder and automatic cuvette changer. Poly(A) and poly(U) were each $2 \times 10^{-5}$ M while Fab was at $1 \times 10^{-5}$ M. Both specific anti-$AU_{Fab}$ and normal Fab were dialyzed against the reaction buffer, which was 0.011 M in $Na^+$. The absorbance decrease at 260 nm was monitored since the ionic conditions employed precluded complications from triple-helix formation (Blake & Fresco, 1966).

The initial absorbance was equal to that expected for the simple mixture of components; that is, Fab had no effect upon the absorbance of the single-stranded polynucleotides. The observed absorbance after 10 h of reaction minus the initial absorbance of the reaction mixture was used as $A_\infty$. Data were analyzed according to a second-order plot as described by Blake & Fresco (1966), or, in the case of the specific Fab reaction, by a semilog plot of $A_\infty - A_t$ vs. time.

Kinetics of triple-helix formation were carried out using poly(A)·poly(U)·poly(U), preformed in 0.1 M $Na^+$ and subsequently diluted to 0.005 M $Na^+$ so that dissociation into poly(A)·poly(U) + poly(U) was complete at room temperature. This polynucleotide mixture was added to a microcuvette containing either anti-$AUU_{Fab}$, anti-$AU_{Fab}$, or normal Fab each in 0.005 M $Na^+$. The final volume was 0.3 mL with Fab and polynucleotide components each at $2 \times 10^{-5}$ M. The absorbance at 260 nm was noted and the reaction commenced upon addition of 9 μL of 1 M $Na^+$ to bring the final $Na^+$ concentration to 0.03 M. Complex formation was monitored as hypochromicity at this wavelength until equilibrium was reached. These initial conditions did not permit complete formation of triple-helix so that an additional 24 μL of stock $Na^+$ was delivered to raise the cation concentration to 0.1 M and thereby permit the reaction to progress further. Protein alone displayed no significant changes in absorption at 260 nm with these increases in cation concentration.

*Immunodiffusion in Gels.* Two-dimensional immunodiffusion experiments (Ouchterlony, 1958) were performed in 0.8% agarose made with 0.15 M NaCl, 0.01 M phosphate buffer (pH 7.4). Purified antibody was tested at 3 mg/mL, while polynucleotides were $1 \times 10^{-6}$ mol/mL. After developing overnight at room temperature, the plates were washed well with buffered saline and then stained for protein.

*Quantitative Precipitin Tests.* Purified antibody (300–350 μg) was reacted with varying amounts of helical polynucleotides in a final volume of 0.5 mL of 0.15 M NaCl, 0.01 M phosphate buffer (pH 7.4). After incubation at 37 °C for 2 h, the mixtures were centrifuged and the precipitates and supernatants separated. These supernatants were analyzed for protein content by the microbiuret procedure (Itzhaki & Gill, 1964). After washing the precipitates one time with 0.5 mL of cold buffered saline, they too were assayed with the protein

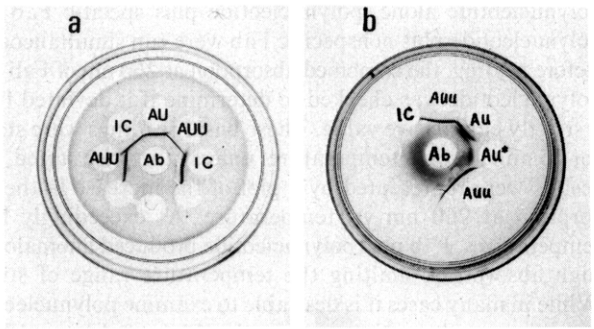


FIGURE 1: Two-dimensional immunodiffusion with either (a) purified anti-AU antibody (Ra73-1) or (b) purified anti-AUU antibody (Ra73-3) at 3 mg/mL. Polynucleotide complexes, poly(A)·poly(U), poly(I)·poly(C), and poly(A)·poly(U)·poly(U), were each at $1 \times 10^{-3}$ M. Poly(A)·poly(U) annealed under conditions which exclude triple-helix contamination is designated AU*.

specific reagent. In later experiments, the $Na^+$ concentration was elevated to 0.3 M.

*Thermal Dissociation of Immune Precipitates.* Poly(A)·poly(U) at $30 \times 10^{-6}$ M and purified anti-AU antibody at $2 \times 10^{-6}$ M (from Ra73-2) were allowed to react for 2 h in 1 mL of buffer at final $Na^+$ concentrations of either 0.0075 M or 0.1 M. These proportions of antibody and antigen were predetermined to provide maximal precipitation and, after vigorous agitation on a vortex mixer, the amount of precipitate was evaluated by monitoring turbidity at 500 nm. The cuvettes were then heated to the stated temperature and the contents allowed to equilibrate for 10 min. After quickly inverting the samples to suspend the precipitate, readings were taken before heating was resumed to reach the next temperature interval. The turbidity of unheated controls did not vary during the course of these experiments.

## Results

*Specificity of the Purified Antibodies.* Immunization with either double-helical or triple-helical polynucleotide complexes of poly(A) and poly(U) elicits ample production of antibodies with the desired specificity but can also induce additional, distinct populations of antibodies with strict specificity either for component single strands or for alternative complexes of the strands. Whether these minor determinants are initially present in the antigen preparation or arise within the experimental animal is not known; however, isolation procedures have been devised which eliminate any "contaminant" antibody populations and provide purified anti-AU antibodies and purified anti-AUU antibodies (Stollar, 1975). Antibodies were isolated from animals immunized with poly(A)·poly(U) using the cross-reactive poly(I)·poly(C) duplex to selectively precipitate only those antibodies directed toward the double helix since this polymer pair cannot form a triple helix (Michelson et al., 1967; Stollar & Stollar, 1970). Anti-AU antibody (Ra73-1) purified in this manner was examined by double diffusion in agarose (Figure 1a). The preparation exhibited a reaction of identity when tested with poly(A)·poly(U) and poly(I)·poly(C) but also gave a reaction of partial identity between either of these double-strand forms and triple-stranded poly(A)·poly(U)·poly(U).

Quantitative precipitin analyses showed that poly(A)·poly(U) and poly(I)·poly(C) precipitated 100% of the purified antibody, while poly(A)·poly(U)·poly(U) precipitated only 70% of it. No reactivity with component single strands was observed and comparable results were obtained using antibodies isolated from two different rabbits (Ra 73-1 and Ra 73-2).

At first inspection both gel diffusion and quantitative precipitin results appeared to indicate the presence of two populations of anti-AU antibodies, a minor fraction possessing strict specificity for the double helix and the major portion reactive with determinants common to both double and triple helix. Succeeding experiments shall be described which ultimately negate this interpretation.

Animals immunized with poly(A)·poly(U)·poly(U) produced antibodies which could be isolated by precipitation with the homologous triple-stranded complex (Stollar & Stollar, 1970). Purified anti-AUU antibody gave a single line with poly(A)·poly(U)·poly(U) and a confluent but less intense line with poly(A)·poly(U), while no reaction was observed with poly(I)·poly(C) (Figure 1b). These characteristics could be interpreted in terms of anti-AUU antibody being nonreactive with double-strand structures but able to detect slight triple-strand "contamination" in poly(A)·poly(U) preparations. Spurious triple-strand formation being chemically impossible in mixtures of poly(I) and poly(C), these preparations of double-helix showed no reactivity with anti-AUU antibodies (Lacour et al., 1968; Stollar, 1973). In order to preclude triple-helix formation, poly(A)·poly(U) was formed at a salt concentration and temperature at which the triple-helical form is not stable (Blake & Fresco, 1966) and a slight excess of poly(A) was included. This preparation was fully reactive with anti-AU antibody but gave only a faint precipitin reaction with anti-AUU antibody (Figure 1b). The line did not merge with the band produced by poly(A)·poly(U)·poly(U).

Thus anti-AU antibodies isolated by virtue of their interaction with double helix were devoid of reactivity with single-stranded structures and were completely reactive with appropriate double-stranded forms, and these same antibodies could recognize determinants on triple-helical poly(A)·poly(U)·poly(U). Antibodies elicited by triple-helical poly(A)·poly(U)·poly(U) and isolated by selective precipitation with this complex displayed distinguishing different specificity characteristics. These were strongly reactive with the homologous triple-stranded complex, unreactive with single-stranded structures, and were not cross-reactive with properly prepared double-helical structures.

*Thermal Dissociation of Poly(A)·Poly(U).* Analysis of the effects of anti-AU antibody combining sites upon the controlled thermal disruption of helical structure provides a sensitive and direct approach to examining the relationship between the binding reaction and helical structure. Absorption readings both at 260 nm and 280 nm for poly(A)·poly(U) in the presence of specific Fab (Ra 73-2) ranging from $1.25 \times 10^{-6}$ to $20 \times 10^{-6}$ M were higher (by 0.008–0.030) than expected for a simple mixture of components. This was not observed for normal Fab; however, being equivalent at both wavelengths, the change was not characteristic of strand separation. Upon subsequent heating, melting profiles were obtained and the level at which these reached a plateau fell in a concentration-dependent manner (Figure 2). This reduction in hyperchromic effect was quite substantial at high Fab levels even considering the possibility that the initial absorbance increases noted at mixing might have resulted in correspondingly diminished potential for thermal hyperchromicity. Thus it appeared that a greater fraction of helix had been prevented from dissociating as an increasing number of sites along its length interacted with Fab. The restraints imposed by this binding were not relieved even at temperatures 30 °C higher than the normal $t_m$. The segments of polynucleotide duplex which were free to disengage did so at increasingly el-

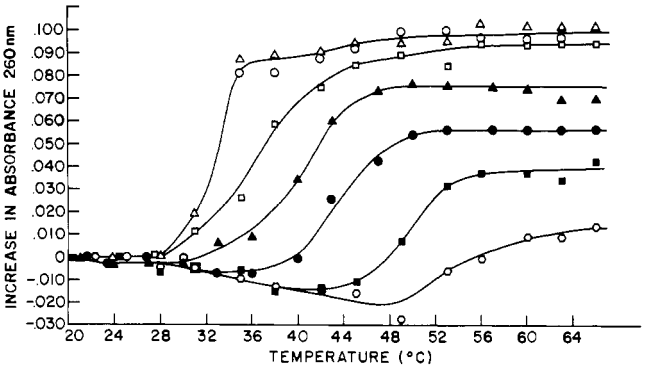


FIGURE 2: Hyperchromicity changes accompanying thermal dissociation of double-helical poly(A)·poly(U) at $2 \times 10^{-5}$ M in 0.006 M $Na^+$. Polynucleotide alone (Δ—Δ), in the presence of normal Fab at $2 \times 10^{-5}$ M (O—O), and in the presence of anti-$AU_{Fab}$ (Ra73-2) at $0.125 \times 10^{-5}$ M (□—□), $0.250 \times 10^{-5}$ M (▲—▲), $0.500 \times 10^{-5}$ M (●—●), $1.00 \times 10^{-5}$ M (■—■), and $2.00 \times 10^{-5}$ M (O—O).

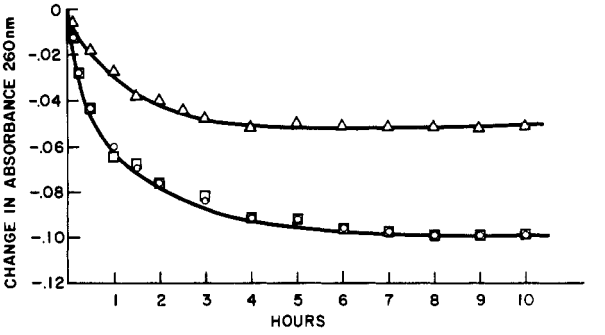


FIGURE 3: Time course of the hypochromic change accompanying poly(A)·poly(U) formation from poly(A) plus poly(U) each at $2 \times 10^{-5}$ M in 0.011 M $Na^+$ at 26.5 °C. Polynucleotide alone (O—O), in the presence of normal Fab at $1 \times 10^{-5}$ M (□—□), and in the presence of anti-$AU_{Fab}$ (Ra73-2) at $1 \times 10^{-5}$ M (Δ—Δ).

evated temperatures as the amount of Fab was raised. Melting was not sharp but may represent the collective dissociation of segments with differing stability. Comparable results were observed for anti-$AU_{Fab}$ isolated from a different rabbit, Ra 73-1.

The specificity of both of the effects ascribed to anti-$AU_{Fab}$ binding was demonstrated by the virtually inert behavior of Fab derived from nonimmunized rabbits. Concentrations as high as 1 mg/mL, when mixed with poly(A)·poly(U), altered neither the extent of thermal hyperchromicity nor its melting temperature (Figure 2). Such solutions remained stable beyond temperatures at which melting occurred, but heating to temperatures which can denature antibody (~70 °C) caused a sharp rise in 260-nm absorbance with no visible turbidity.

An anomalous decrease in absorbance preceded the hyperchromicity due to strand separation at the higher levels of specific Fab and was not seen with normal Fab. This might represent a reversal of the initial increase observed upon mixture of anti-$AU_{Fab}$ and poly(A)·poly(U) but the physicochemical basis of these changes has not been identified.

Destabilization of helical structure comes chiefly from charge repulsions and entropy gained in conformational disorder. The ability of the anti-AU combining site to contravene thermal pertubances is indicative of its ability to envelop both strands and physically restrain free movement. It is deemed unlikely that such a site would possess the critical arrangement of functional groups necessary to function as an efficient counterion to screen negatively charged phosphate groups or to increase hydrophobic stacking forces between vertically adjacent bases.

*Thermal Dissolution of Antibody–Polynucleotide Immune Precipitates.* It was of interest to determine whether the general stabilizing features displayed by the binding of specific Fab fragments would also pertain to whole divalent antibody upon its combination with structured polynucleotides. The insoluble cross-linked complexes which result from this interaction obscure direct observation of the conformational changes which polynucleotides may undergo; however, the amount of immune precipitate can be utilized as an indicator for the presence of both intact helix and antibody sites. Progressive heating of preformed anti-AU-poly(A)·poly(U) immune precipitates revealed that, whereas poly(A)·poly(U) alone in 0.0075 M $Na^+$ was completely separated at 40 °C, the immune complex did not dissociate fully until 75 °C (Table I). A similar effect was noted at 0.1 M $Na^+$. It is interesting to note that antibody was not irreversibly denatured but regained binding ability even after exposure to 75 °C.

TABLE I: Thermal Dissolution of Immune Precipitates Formed by Anti-AU Antibody and Poly(A)·Poly(U).[a]

| Temp (°C) | % precipitate (0.0075 M $Na^+$) | % precipitate (0.1 M $Na^+$) |
|---|---|---|
| 25 | 100 | 100 |
| 55 | 98 | 87 |
| 65 | 41 | 67 |
| 75 | 0 | 1 |
| 25 | 100[b] | 91[c] |
| $t_m$ (polynucleotide alone) | 34 °C | 56 °C |

[a] The amount of precipitate was evaluated by monitoring the turbidity at 500 nm. Values at the different temperatures were compared with the amount of precipitate which had formed after incubation of reactants for 2 h at 25 °C. [b] Reading taken after remaining at 25 °C overnight. [c] Reading taken after remaining at 25 °C for 45 min.

Thus the helical segments to which antibody bound were prevented from separating at the normal $t_m$ until, at still higher temperatures, either these regions or the antibody which stabilized them denatured, effectuating disassembly of the precipitate.

*Kinetics of Poly(A)·Poly(U) Formation.* At any particular temperature in the melting curve of free polynucleotide there exists a dynamic equilibrium with constant dissociation and reassociation of base pairs. Reassociation might well have been modified by the presence of anti-$AU_{Fab}$ during melting and, since the potential of these sites to effect de novo helix formation offered some interesting possibilities, the kinetics was studied. The rate of annealing of the homopolymer pair was determined in the presence and absence of specific Fab, employing conditions similar to those of the melting experiments (Figure 3).

Second-order rate constants for the reaction of polynucleotide alone or with normal Fab were both $k_2$(apparent) = 22.8 L $mol^{-1}$ $s^{-1}$. Curiously, in the presence of anti-$AU_{Fab}$, the final extent of base pairing reached only 50% of maximum and the fraction of polynucleotide which annealed followed first-order kinetics with $k_1$(apparent) = $2.13 \times 10^{-4}$ $s^{-1}$.

To investigate the nature of poly(A)·poly(U) formed under the influence of anti-$AU_{Fab}$, the melting profile for each of these reaction mixtures was determined (Figure 4). The duplex polynucleotide formed alone or in the presence of normal Fab produced comparable melting curves while the mixture which

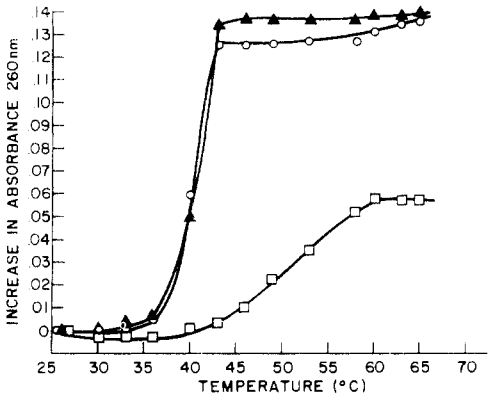


FIGURE 4: Hyperchromicity changes accompanying thermal dissociation of the products formed in the polynucleotide association reactions described in Figure 3. Polynucleotide complex formed and tested in 0.011 M $Na^+$ alone (▲—▲), polynucleotide complex formed and tested in the presence of $1 \times 10^{-5}$ M normal Fab (O—O) and polynucleotide complex formed and tested in the presence of $1 \times 10^{-5}$ M anti-$AU_{Fab}$ (Ra73-2) (□—□).

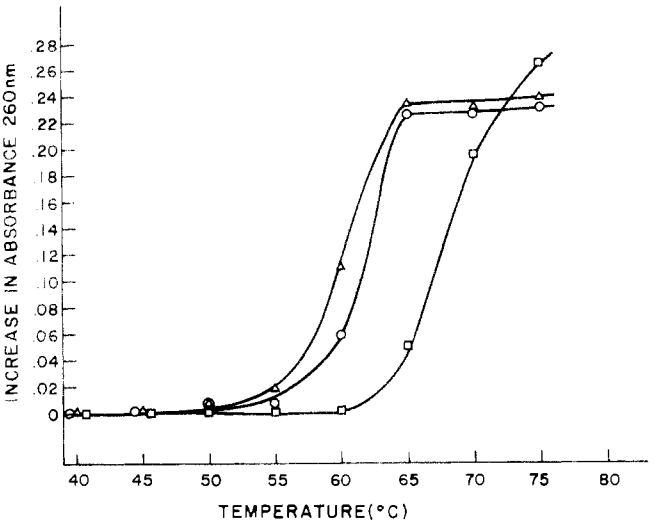


FIGURE 5: Hyperchromicity changes accompanying thermal dissociation of triple-helical poly(A)·poly(U)·poly(U) at $2 \times 10^{-5}$ M in 0.15 M $Na^+$. Polynucleotide alone (O—O), in the presence of $1 \times 10^{-5}$ M anti-$AU_{Fab}$ (Ra73-1) (△—△) and in the presence of $1 \times 10^{-5}$ M anti-$AUU_{Fab}$ (Ra73-3) (□—□).

included specific anti-$AU_{Fab}$ displayed distinguishing features. Strand separation occurred at elevated temperatures; however, the temperature span over which melting took place was greater than is normally observed. Further, all of the base pairs which formed during the anti-$AU_{Fab}$ modulated annealing process were dissociated upon heating, since the extent of hypochromicity observed at the end of the kinetics run was completely recovered as hyperchromicity during melting. This is in contrast with the inability to achieve complete hyperchromicity when anti-$AU_{Fab}$ reacts with preformed poly(A)·poly(U) (Figure 2).

It is believed that the annealing process for long chain polynucleotides consists of primary, indiscriminate base-pairing interactions at multiple sites along the chain, followed by numerous dissociation–reassociation steps or a lateral slippage mechanism which optimizes base pairing (Felsenfeld, 1958; Pörschke & Eigen, 1971; Craig et al., 1971). While anti-$AU_{Fab}$ binding sites could be construed in terms of a template which might markedly facilitate base-pair formation, no evidence of this was found, since the half-time of the overall reaction was increased. Less than complete annealing would result if anti-$AU_{Fab}$ molecules bound to helical sites as they appeared and hindered further rearrangements of the strands. The melting profile demonstrated that those polynucleotide segments which annealed were stabilized by bound Fab and dissociated in a noncooperative manner.

*Thermal Dissociation of Triple Helix.* Examination of the effects of specific antibody binding upon the thermal transitions of triple helical poly(A)·poly(U)·poly(U) were carried out at high and low cation concentration since melting of this complex can proceed in either one or two steps depending upon the $Na^+$ concentration (Stevens & Felsenfeld, 1964; Massoulie et al., 1964; Michelson et al., 1967; Riley et al., 1966). While both anti-AU and anti-AUU were shown to bind the triple helix (Figure 1a,b), this approach provided an opportunity to draw distinctions in the mode of interaction based upon direct observation of their involvement with individual strands of helix.

*Monophasic Dissociation.* Upon heating in 0.15 M $Na^+$, poly(A)·poly(U)·poly(U) separated directly into its constituent strands ($t_m$ = 61 °C)

$$AUU \xrightarrow{t_m} A + U + U$$

to produce a single hyperchromicity transition (Figure 5) and normal Fab at $1 \times 10^{-5}$ M did not influence these melting characteristics. Anti-$AU_{Fab}$ (Ra 73-1) at the same concentration had a minimal effect shifting the curve about 1–2 °C toward lower temperature. Anti-$AUU_{Fab}$, on the other hand, was distinguished by its ability to substantially stabilize the triple helix, melting occurring at a temperature 7 °C higher than controls (Figure 5). The monophasic nature of the hyperchromic change indicated that all three strands disengaged simultaneously when anti-$AUU_{Fab}$ was bound. It was difficult to assign an accurate $t_m$ for this antibody modified melting transition since there were several indications that an anomalous reaction occurs between the triple helix and specific Fab at the higher temperatures. In the case of triple helix combined with homologous anti-$AUU_{Fab}$, 260-nm absorbance increased beyond the limits expected for triple strand melting alone (Figure 5).

These findings established that anti-$AUU_{Fab}$ could indeed stabilize the three-stranded structure while anti-$AU_{Fab}$ displayed no similar capability, failing even to delay melting of the double-helical component of the structure. This opened questions as to the nature and degree of anti-$AU_{Fab}$ interaction with poly(A)·poly(U)·poly(U), under these conditions.

Some pertinent observations were made relating to the extent of polynucleotide renaturation in each of the reaction mixtures. Upon standing overnight at room temperature, the cuvettes that contained heat dissociated poly(A) plus 2 poly(U) alone, or in the presence of normal Fab completely renatured to form triple helix as witnessed by a total drop in 260-nm absorbance back to the level recorded before melting. In the presence of anti-$AU_{Fab}$ regain of hypochromicity was only half complete, a result which indicated that this protein retained at least partial binding activity after heating and that its interaction interfered in a specific manner to prevent proper annealing of triple helix. Further indication of anomalous interaction between triple helix and anti-$AUU_{Fab}$ after heating was found, since little change in its absorbance at 260 nm was noted upon standing overnight at room temperature.

*Biphasic Dissociation.* Poly(A)·poly(U)·poly(U) is a stable structure in 0.031 M $Na^+$ at room temperature but in contrast to the experiments performed in 0.15 M $Na^+$, a biphasic transition occurs upon heating under these reduced ionic conditions (Stevens & Felsenfeld 1964; Massoulie et al., 1964;

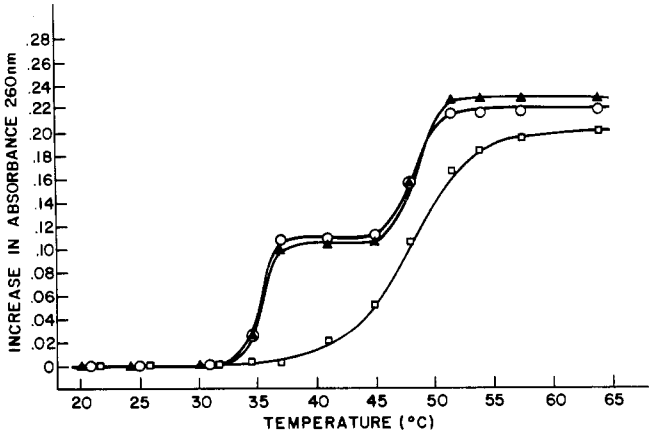


FIGURE 6: Hyperchromicity changes accompanying thermal dissociation of triple-helical poly(A)·poly(U)·poly(U) at $2 \times 10^{-5}$ M in 0.031 M $Na^+$. Polynucleotide alone (▲—▲), in the presence of normal Fab at $2 \times 10^{-6}$ M (○—○), in the presence of anti-$AUU_{Fab}$ (Ra73-3) at $2 \times 10^{-6}$ M (□—□).

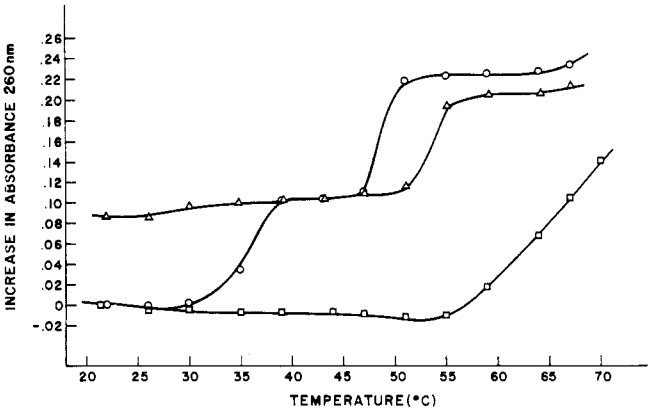


FIGURE 7: Hyperchromicity changes accompanying thermal dissociation of triple-helical poly(A)·poly(U)·poly(U) at $2 \times 10^{-5}$ M in 0.031 M $Na^+$. Polynucleotide alone (○—○), in the presence of anti-$AU_{Fab}$ (Ra73-1) at $1 \times 10^{-5}$ M (△—△), and in the presence of anti-$AUU_{Fab}$ (Ra73-3) at $1 \times 10^{-5}$ M (□—□).

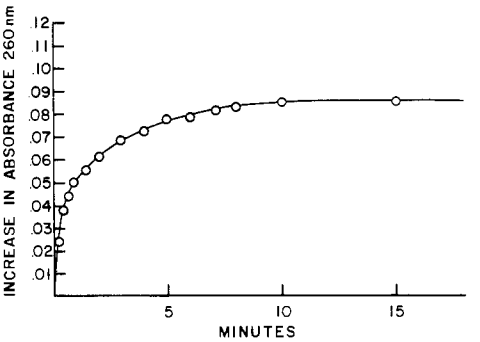


FIGURE 8: Time course of the hyperchromic change observed upon adding anti-$AU_{Fab}$ (Ra73-1) at a final concentration of $1 \times 10^{-5}$ M to poly(A)·poly(U)·poly(U) at $2 \times 10^{-5}$ in 0.031 M $Na^+$.

Riley et al., 1966; Michelson et al., 1967). Two distinct increases in absorbance at 260 nm occurred as heating progressed and these corresponded to an initial release of the third strand ($t_1$ = 35 °C) followed by separation of the remaining poly(A)·poly(U) double helix ($t_2$ = 48 °C). The basic pattern observed for

$$AUU \xrightarrow{t_1} AU + U \xrightarrow{t_2} A + U + U$$

is shown in Figure 6 along with profiles obtained with inclusion of either normal Fab or anti-$AUU_{Fab}$, at $2 \times 10^{-6}$ M. While normal Fab effected no change in the melting profile, it appeared that anti-$AUU_{Fab}$ retarded release of the poly(U) chain and melting occurred in one step rather than in two distinct transitions. The dissociated polynucleotide chains in each of these reaction mixtures annealed to give 50% of the maximum hypochromicity after cooling at room temperature for 1 h. No further changes were noted after incubation at 4 °C overnight indicating that helix formation was not complete under these conditions.

To further elucidate the relationship of selective antibody binding with the low ionic strength interconversions of poly(A)·poly(U)·poly(U), five times more anti-$AUU_{Fab}$ was added, and in addition, anti-$AU_{Fab}$ (Ra73-1) was also tested at $1 \times 10^{-5}$ M. Normal Fab at this higher concentration did not affect the biphasic melting of the three-stranded structure while both anti-$AU_{Fab}$ and anti-$AUU_{Fab}$ each caused dramatic but strikingly different alterations in these thermal transitions (Figure 7).

Increasing the amount of anti-$AUU_{Fab}$ fivefold (Figure 7) gave rise to a substantial elevation in melting temperature, demonstrating that the extent of stabilization of the triple helix was dependent upon anti-$AUU_{Fab}$ concentration. More importantly, the onset of melting was delayed beyond temperatures at which both phases of hyperchromicity had already occurred in the normal Fab control reaction. Thus, the anti-$AUU_{Fab}$ binding sites exert their effect upon all three strands simultaneously.

Anti-$AU_{Fab}$ (Ra73-1) affected the biphasic melting profile in two ways. The mixture containing these anti-double-helical binding sites became hyperchromic before the melting experiment was begun. That is, addition of this antibody appeared to cause a rapid release of the third strand at 22 °C, a temperature at which the triple helix alone is stable in 0.031 M $Na^+$. As heating was commenced, no absorbance changes were noted in the region normally associated with poly(U) dissociation, while, at higher temperatures, anti-$AU_{Fab}$ caused an anticipated stabilization and increased $t_m$ for the remaining double strand (Figure 7).

The time course of poly(U) elimination was followed immediately upon admixture of anti-$AU_{Fab}$ and poly(A)·poly(U)·poly(U) (Figure 8). Control reactions which contained polynucleotide alone, normal Fab, or anti-$AUU_{Fab}$ displayed no absorbance increase in this time span. Further, the effect was observed with anti-$AU_{Fab}$ from Ra73-1 as well as from Ra73-2.

The effects of Fab upon both mono- and biphasic hyperchromicity transitions of the triple-helical structure indicate that anti-AUU binding sites envelop all three strands and this interaction interferes with their thermal disassembly. The experiments distinguish anti-$AU_{Fab}$ binding in that reaction occurs not with intact triple helix but rather with its double-helical component and only when conditions permit dissociation of the extra poly(U) strand.

*Triple Helix Formation.* A feasible mechanism of strand expulsion would follow if binding of anti-$AU_{Fab}$ to double-helical determinants could sterically obstruct the poly(U) strand from entwining within the major groove (Arnott & Bond, 1973; Stollar & Raso, 1974). Triple-helix formation was initiated in the presence of specific Fab to verify such blockage.

Equimolar amounts of poly(A)·poly(U) and poly(U) at 25 °C in 0.005 M $Na^+$ can be made to associate to produce triple

TABLE II: Effects of Fab upon Triple Helix Formation, Poly(A)·Poly(U) + Poly(U) ⇌ Poly(A)·Poly(U)·Poly(U).

| Protein included ($2 \times 10^{-5}$ M) | Hypochromic change at 260 nm (0.03 M $Na^+$) | (0.10 M $Na^+$) |
|---|---|---|
| Buffer alone | 0.044 ± 0.007[a] | 0.062 ± 0.015 |
| Normal Fab | 0.059 ± 0.005 | 0.118 ± 0.005 |
| Anti-$AUU_{Fab}$ | 0.044 ± 0.002 | 0.120 ± 0.014 |
| Anti-$AU_{Fab}$ | 0.004 ± 0.003 | 0.119 ± 0.015 |

[a] Average of three experiments.

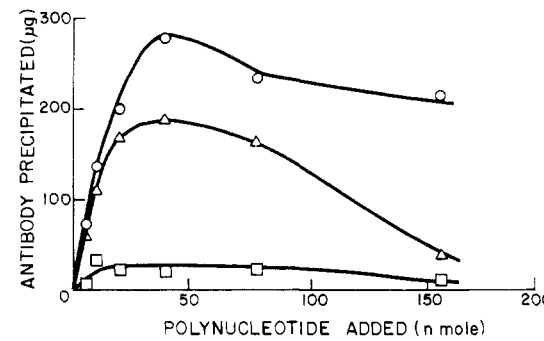


FIGURE 9: Quantitative precipitin reactions at high $Na^+$ concentration. A constant amount (300 μg) of anti-AU antibody (Ra73-2) was mixed with varying amounts of polynucleotide in a 0.5-mL reaction volume and incubation proceeded for 2 h at 37 °C. Antibody in the washed precipitates was evaluated with microbiuret reagent. Poly(A)·poly(U) in 0.30 M NaCl, 0.01 M phosphate buffer (○—○), poly(A)·poly(U)·poly(U) in 0.15 M NaCl, 0.01 M phosphate buffer (△—△), and poly(A)·poly(U)·poly(U) in 0.30 M NaCl, 0.01 M phosphate buffer (□—□).

helix by raising the cation concentration to 0.03 M or higher. Annealing of the poly(U) strand is signaled by a decrease in the absorbance at 260 nm and the influence of specific Fab preparations upon this reaction was examined by including the protein before raising the $Na^+$ concentration (Table II). In the presence of buffer alone, or with normal Fab, an increase of $Na^+$ to 0.03 M resulted in partial annealing of the triple helix, and anti-$AUU_{Fab}$ displayed no interference with this, while anti-$AU_{Fab}$ effected almost total blockage of the interaction. These hypochromic changes were rapid and the values did not change during a 30-min observation period. A further rise in cation concentration to 0.1 M caused an additional hypochromic change in all reaction mixtures. The extent of triple-helix formation appeared to be complete in the mixtures containing either normal Fab, anti-$AU_{Fab}$, or anti-$AUU_{Fab}$ while all the potential base pairing did not occur in the absence of protein.

The effective interference provided by anti-$AU_{Fab}$ was compatible with the concept of sites which span both strands to occlude the major groove and prevent annealing of the third strand. Anti-$AUU_{Fab}$ showed no similar effect which is consistent with its inability to react with the double helix. It is interesting that the block observed in the anti-$AU_{Fab}$ mixture at low ionic strength was almost fully released at the higher cation concentration. The fact that there was no indication of reversal of the Fab blockage with time suggests that its binding affinity for poly(A)·poly(U) is greater than that of poly(U) at 0.03 M $Na^+$. This situation might be reversed at 0.1 M $Na^+$ since the association constant of the third strand would be greatly increased at higher cation concentrations (Felsenfeld & Rich, 1957). These results parallel the previous finding that anti-$AU_{Fab}$ could promote elimination of the third strand from a triple helix at 0.031 M $Na^+$, but not at 0.15 M $Na^+$.

*Quantitative Precipitation of Triple Helix with Anti-AU Antibody.* In view of the apparent ability of anti-$AU_{Fab}$ to render triple-helical poly(A)·poly(U)·poly(U) into its component double helix plus a strand of poly(U), it became important to reevaluate the precipitation of triple helix by these antibodies (Figure 1). Precipitation curves were obtained using anti-AU antibody (Ra73-2) with poly(A)·poly(U) run at 0.3 M $Na^+$ and with poly(A)·poly(U)·poly(U) run at the conventional 0.15 M $Na^+$ concentration as well as at 0.3 M $Na^+$ (Figure 9). This rise in cation concentration obliterates any cross-reactivity between anti-AU antibodies and the triple-helical preparation at 37 °C. Similar results were obtained if, instead of raising the cation concentration, all reactions were run with 0.15 M $Na^+$ but at 4 °C. These results are interpreted to mean that anti-AU antibodies have absolute specificity for the double helix. Under certain ionic conditions, however, spontaneous and reversible unwinding of the poly(U) chain occurs so that limited double-stranded regions are fleetingly present in the triple helix. Anti-AU antibody can then react with these segments, perhaps causing further unwinding, and ultimately precipitation occurs.

When the triple-helical preparation was reacted with an excess of anti-AU antibodies in 0.15 M $Na^+$ at 37 °C, all the polynucleotide was found in the precipitate. An equivalent of poly(U) was not found in the supernatant as might have been expected if the antibody had caused complete elimination of the poly(U) strand. Thus, it seems likely that, under these conditions, the third strand retained at least some contact points with the double helix.

## Conclusion

The series of complex melting profiles obtained for poly(A)·poly(U) with anti-$AU_{Fab}$ added in increasing concentration has been interpreted in terms of two related phenomena. The decreasing level of hyperchromicity is thought to reflect the cumulative number of sites in the helix which are interacting directly with Fab and are therefore unable to separate. Polynucleotide segments between these contact points, which ultimately dissociated, displayed increasingly higher $t_m$ values as the interval between Fab sites became shorter and more stabilized.

Progressive effects were noted as the stoichiometry increased from 12.5 to 100 Fab molecules added per stretch of 100 base pairs (Figure 2). Such a segment of polynucleotide is 300 Å in length (Arnott & Bond, 1973) and, according to the dimensions of a Fab fragment (Valentine & Green, 1967; Green, 1969), could accommodate at most 7 such molecules closely packed in a side-by-side array. Again, because of steric considerations, no more than 2 or 3 Fab molecules could be expected to be directly opposite each other when surrounding the same point in the axis of a helix. Thus, the number of Fab's bound to a 100 base pair stretch of helix would be in the range of 14–21.

The relationship between the depression in the final hyperchromicity level and the amount of Fab added is consistent with a simple equilibrium titration of available helix sites. If, however, the laws of reversible equilibria pertain for these reaction conditions, specific Fab molecules must be constantly binding, dissociating, and recombining with these helical segments before they have a chance to melt. This holds even at temperatures far beyond the $t_m$ of free poly(A)·poly(U). Rates of association for antibody combining sites and haptenic determinants are typically very fast, with $k_1$ values in the range of $10^7$ to $10^8$ $M^{-1}$ $s^{-1}$ (Froese, 1968; Pecht et al., 1972). The

rate of dissociation of a double helical oligoribonucleotide consisting of 10 base pairs is about 800 $s^{-1}$, in the temperature vicinity of its $t_m$ (Pörschke & Eigen, 1971). Calculation of reaction velocities using these figures along with the concentration of reactants leads to the conclusion that melting of the helical segment and the binding of Fab to polynucleotide occur at comparable speeds.

It seems probable that this discrepancy is merely a reflection of the unique character of a continuous helix with Fab molecules specifically bound at periodic intervals along its length. If one considers that each Fab binding event stabilizes a helical nucleus, it is not surprising that such interactions would have far-reaching effects on the rate of dissociation of adjacent base-paired segments even at elevated temperatures (Pörschke & Eigen, 1971; Craig et al., 1971).

Compared with Fab fragments, whole bivalent anti-AU antibody appeared more efficient in stabilizing polynucleotide complexes. Antibody combining sites at a multiplicity of 13.3 per 100 base pairs were used to form the precipitates for these studies. This relatively low ratio produced substantial stabilization of the helix compared with the minimal attenuation provided by a similar amount of anti-$AU_{Fab}$ (indicated by the first anti-$AU_{Fab}$ curve of Figure 2). This difference is not surprising in light of the binding advantage provided by multivalency considerations which apply for reactions of bivalent antibody with the repeating sites on poly(A)·poly(U) (Hornick & Karush, 1972; Crothers & Metzger, 1972). The insensitivity of heat-induced solubilization to ionic conditions, in contradistinction to the strong cation dependence of helix stability, suggests that the primary event leading to dissociation of these precipitates is thermal disruption (denaturation) of antibody binding sites.

The blockage of strand separation provided by anti-$AU_{Fab}$ fragments and whole antibody is in full accord with the conception of individual binding sites with the capacity to encompass and interact with both strands simultaneously. This view was initially arrived at through specificity studies using different combinations of polynucleotide chains to construct various helical forms (Stollar & Raso, 1974; Stollar, 1975).

That anti-AU binding sites influence both the mechanisms of helix formation and nature of the final product is reflected in the change of kinetic order of reaction in conjunction with the reduced extent of base pairing which occurs when specific Fab is present. According to relaxation kinetic studies using oligoadenylate–oligouridylate complexes (Pörschke & Eigen, 1971), helix formation proceeds via formation of a critical nucleus, consisting of 3 base pairs, to which additional base pairs may be consecutively added in a rapid, cooperative manner. Duplex formation involving long chain polymers is further complicated by the fact that nuclei may form at several random points along the chain (Pörschke & Eigen, 1971) and allowance must therefore be made for a rearrangement mechanism to correct any loops or unpaired ends which initially occur (Felsenfeld, 1958). It seems probable that anti-$AU_{Fab}$ affects helix formation at the level of base-pair addition or strand rearrangement rather than at the initiation of nuclei. Observed changes of reaction order as well as the reduced extent of hypochromicity could result if the rate of helix growth was impeded and elimination of unpaired regions was curtailed by anti-$AU_{Fab}$ interactions.

The influence exerted by the presence of Fab during initiation of helix formation may be quite different from that which bound Fab has upon the rate of reannealing partially dissociated sections of preformed helix. This latter situation applies during the melting experiments. Strands are already in proper register and Fab binding, by both preserving this alignment and stabilizing nuclei, would communicate its effect along large portions of helix. On the other hand, when free disoriented polynucleotide chains begin to associate by random pairings from solution, these Fab molecules could maintain misalignments which initially develop. Indeed, the altered melting characteristics displayed by these modified duplex polynucleotides indicate a less stabilized and completely dissociable structure (Figure 4).

Combining sites specific for triple-helical determinants stabilize the three-stranded structure in a manner analogous to the observed stabilization of poly(A)·poly(U) by anti-AU sites. Their effect upon the thermal dissociations of triple helices indicates that all three strands are restrained as a unit. A substantial increase in melting temperature of the triple helix was effectuated by anti-$AUU_{Fab}$ under ionic conditions which favor simultaneous dissociation of the three chains (Figure 5). At lower ionic strength anti-$AUU_{Fab}$ changed the biphasic mode of melting into a single continuous transition, a result consistent with combining sites encompassing all three polynucleotide chains (Figures 6 and 7). Lastly, under these conditions, specific Fab at high concentration delayed the onset of melting beyond the normal melting temperatures of both the primary AUU → AU + U subsequent AU → A + U dissociations.

Anomalies in the 260-nm absorbance encountered at the high temperature range necessary for melting triple helix plus anti-$AUU_{Fab}$ obscured observation of a final plateau for most of these profiles. Thus, it is not known if a progressive reduction of these plateau levels accompanies the Fab concentration dependent increase in $t_m$. The curve in Figure 6, however, suggests that this feature, too, may be analogous to that demonstrated for the poly(A)·poly(U)–anti $AU_{Fab}$ system.

The discovery that anti-$AU_{Fab}$ promoted release of the poly(U) chain from the triple-helical complex was totally unexpected and opened a new dimension for the interpretation of interactions between antibodies and polynucleotides. Antibody binding cannot be thought of as a strictly passive attachment of protein to immutable antigen, but rather as a dynamic reaction which potentially can cause drastic changes in the structure of the antigen. The anti-apomyoglobin–myoglobulin system (Crumpton, 1966) is another instance in which antibody was found to play an active role in changing the nature of the antigen. In this case, antibodies and Fab directed against the apoprotein effectuated dissociation of the prosthetic heme group from the holoprotein.

One plausible mechanism for the effect produced by anti-$AU_{Fab}$ could proceed with its binding to triple helix to produce a conformational change which expels the extra strand of poly(U). The cumulative data, however, indicate that the anti-$AU_{Fab}$ acts by changing the equilibria of normally occurring polynucleotide intraconversions. Following the initial hyperchromic change, subsequent melting was characteristic of double helix with stabilizing Fab bound to it (Figure 7). This immediately suggested that the poly(U) elimination phenomenon entailed a competition between two reversible equilibrium reactions involving double-helical oligo(A)·oligo(U) segments. Such sites could react either with an increment of poly(U) to form a triple-helical region or with anti-$AU_{Fab}$ but not with both simultaneously. This hypothesis was supported by the finding that poly(U) did not anneal to double helix which had been preexposed to anti-$AU_{Fab}$ at a $Na^+$ concentration of 0.03 M (Table II). Since the reactions are mutually exclusive, the one which predominates must be determined by their relative binding strengths for the duplex. Under conditions of low ionic strength or high temperature, the affinity of poly(U) for poly(A)·poly(U) decreases and

anti-$AU_{Fab}$ can effectively compete for these double-helical sites. Thus, anti-$AU_{Fab}$ mediated poly(U) elimination was observed at room temperature in 0.031 M $Na^+$ but not at 0.15 M $Na^+$. Similarly, binding of anti-$AU_{Fab}$ to poly(A)·poly(U) prevented annealing of poly(U) at 0.031 M $Na^+$, but, when the ionic strength was raised, poly(U) addition was favored over Fab binding and triple-helix formation proceeded.

It was eventually realized that the apparent precipitation of poly(A)·poly(U)·poly(U) with anti-AU antibody was actually a manifestation of the same poly(U) dissociation phenomenon, exposed double-helical sites being the determinants recognized by antibody. Whole bivalent anti-AU antibody can functionally increase its binding effectiveness over Fab when interacting with the repeating sites of the poly(A)·poly(U) structure (Hornick & Karush, 1972; Crothers & Metzger, 1972). It is consistent, therefore, that poly(U) displacement by whole antibody as determined by precipitability can occur at higher ionic strengths and lower temperatures. Thus, 0.15 M $Na^+$ was not sufficient to prevent antibody displacement of poly(U) as it was in the case of monovalent Fab. The balance was tipped toward triple-helix formation by raising the cation concentration to 0.3 M or, if at 0.15 M $Na^+$, the temperature was lowered to 4 °C.

Antibodies directed toward poly(I)·poly(C) display similar properties to antibodies elicited by poly(A)·poly(U) (Lacour et al., 1968; Stollar, 1970, 1975; Stollar & Stollar, 1970) reaffirming the close conformational correspondence between these two helical complexes (Arnott et al., 1973). Indeed, by double-diffusion experiments the anti-IC antibodies have also been shown to be reactive with triple-helical poly(A)·poly(U)·poly(U) (Lacour et al., 1968). Preliminary studies with two anti-IC sera have shown that increasing the salt concentration of the medium to 0.3 M largely or totally abrogated this precipitation while causing no reduction in the reactions with double-helical poly(I)·poly(C) or poly(A)·poly(U).

Previously, this type of cross-reactivity had been attributed to either small amounts of "contaminating" double-helical structure in these poly(A)·poly(U)·poly(U) preparations (Stollar, 1973, 1975) or to genuine interaction of anti-AU binding sites with complementary regions of the intact triple helix (Stollar & Raso, 1974). This later hypothesis was prompted in part by x-ray crystallography studies which showed that the basic configuration of the poly(A)·poly(U) double helix is retained in the structure of the triple helix (Arnott & Bond, 1973). In both of these instances, one must postulate double-helical determinants in the triple-helix preparation which are reactive with anti-AU antibodies yet which, when injected into rabbits, fail to elicit any detectable populations of antibodies cross-reactive with double-helical poly(A)·poly(U) or poly(I)·poly(C). A more consistent picture develops if the poly(U) elimination phenomenon is indeed the source of the cross-reactivities which have been observed previously and in this study (Lacour et al., 1968; Stollar & Raso, 1974; Stollar & Stollar, 1970).

If various different double-helical polynucleotide complexes share several closely related and interchangeable configurations, these equilibria would be subject to pressures similar to those observed for the antibody effected triple- to double-helix interconversion. Since the stereochemical features of a given duplex constitute the basis of immunochemical recognition, the extent of cross-reaction with a heterologous anti-double-helix antibody could be a measure of the effectiveness in shifting these equilibria. The helix would assume an otherwise minor but allowable conformational state to achieve maximum complementarity with the heterologous combining site.

Note Added in Proof

After completion of this manuscript we became aware of a related study using antibodies to poly(I)·poly(C) (Guigues, M., & Leng, M. (1976) *Eur. J. Biochem. 69,* 615–624).

References


Arnott, S., & Bond, P. J. (1973) *Nature (London), New Biol. 244,* 99–101.

Arnott, S., Hukins, D. W. L., Dover, S. D., Fuller, W., & Hodgson, A. R. (1973) *J. Mol. Biol. 81,* 107–122.

Barbu, E., & Panijel, J. (1960) *C.R. Acad. Sci. 250,* 1382–1384.

Blake, R. D., & Fresco, J. R. (1966) *J. Mol. Biol. 19,* 145–160.

Butler, V. P., Beiser, S. M., Erlanger, B. F., Tanenbaum, S. W., Cohen, A., & Bendich, A. (1962) *Proc. Natl. Acad. Sci. U.S.A. 48,* 1597–1602.

Craig, M. E., Crothers, D. M., & Doty, P. (1971) *J. Mol. Biol. 67,* 383–401.

Crothers, D. M., & Metzger, H. (1972) *Immunochemistry 9,* 341–357.

Crumpton, M. J. (1966) *Biochem. J. 100,* 223–232.

Erlanger, B. F., & Beiser, S. M. (1964) *Proc. Natl. Acad. Sci. U.S.A. 52,* 68–74.

Felsenfeld, G. (1958) *Biochim. Biophys. Acta 29,* 133–144.

Felsenfeld, G., & Rich, A. (1957) *Biochim. Biophys. Acta 26,* 457–468.

Froese, A. (1968) *Immunochemistry 2,* 135–143.

Goldfarb, D. M. (1968) *Immunology of Nucleic Acids,* Nauka, Moscow.

Green, N. M. (1969) *Adv. Immunol. 11,* 1–30.

Hornick, C. L., & Karush, F. (1972) *Immunochemistry 9,* 325–340.

Itzhaki, R. F., & Gill, D. M. (1964) *Anal. Biochem. 9,* 401–410.

Lacour, F., Michelson, A. M., & Nahon, E. (1968) in *Nucleic Acids in Immunology* (Plesicia, O. J., & Braun, W., Eds.) pp 32–46, Springer-Verlag, New York, N.Y.

Lacour, F., Nahon-Merlin, E., & Michelson, M. (1973) *Curr. Top. Microbiol. Immunol. 62,* 1–39.

Levine, L., & Stollar, B. D. (1968) *Prog. Allergy 12,* 161–191.

Levine, L., & Van Vunakis, H. (1966) in *Antibodies to Biologically Active Molecules* (Cinadar, B., Ed.) pp 25–60, Pergamon Press, Oxford.

Massoulie, J., Blake, R., Kotz, L., & Fresco, J. (1964) *C.R. Acad. Sci. 259,* 3104–3107.

Michelson, A. M., Massoulie, J., & Guschlbauer, W. (1967) *Prog. Nucleic Acid Res. Mol. Biol. 6,* 83–141.

Nahon, E., Michelson, A. M., & Lacour, F. (1967) *Biochim. Biophys. Acta 149,* 127–139.

Ouchterlony, O. (1958) *Prog. Allergy 5,* 1–78.

Panijel, J., Souleil, C., & Cayeux, P. (1966) *Science 152,* 773–775.

Pecht, I., Givol, D., & Sela, M. (1972) *J. Mol. Biol. 68,* 241–247.

Plescia, O. J., & Braun, W. (1967) *Adv. Immunol. 6,* 231–252.

Plescia, O. J., Braun, W., Imperato, S., Cora-Block, E., Jaroskova, L., & Schimbor, C. (1968) in *Nucleic Acid in Immunology* (Plescia, O. J., & Braun, W., Eds.) pp 5–17, Springer-Verlag, New York, N.Y.

Pörschke, D., & Eigen, M. (1971) *J. Mol. Biol. 62,* 361–381.

Porter, R. R. (1959) *Biochem. J. 73,* 119, 126.

Putnam, F. W., Tan, M., Lynn, L. T., Easley, C. W., & Migita,

S. (1962) *J. Biol. Chem. 237,* 717-726.
Riley, M., Maling, B., & Chamberlin, M. J. (1966) *J. Mol. Biol. 20,* 359-389.
Schwartz, E. F., & Stollar, B. D. (1969) *Biochem. Biophys. Res. Commun. 35,* 115-120.
Stevens, C. L., & Felsenfeld, G. (1964) *Biopolymers 2,* 293-314.
Stollar, B. D. (1970) *Science 169,* 609-611.
Stollar, B. D. (1973) in *The Antigens,* (Sela, M., Ed.) Vol. I, pp 1-85, Academic Press, New York, N.Y.
Stollar, B. D. (1975) *CRC Crit. Rev. Biochem.,* 45-69.
Stollar, B. D., & Raso, V. (1974) *Nature (London) 250,* 231-234.
Stollar, B. D., & Stollar, V. (1970) *Virology 42,* 276-280.
Valentine, R. C., & Green, N. M. (1967) *J. Mol. Biol. 27,* 615-617.

# Methylation and Capping of RNA Polymerase II Primary Transcripts by HeLa Nuclear Homogenates†

Yoram Groner,* Eli Gilboa, and Haim Aviv

ABSTRACT: HeLa nuclear homogenates incubated in vitro incorporate [$\beta$-$^{32}$P]ATP and *S*-[*methyl*-$^{3}$H]adenosylmethionine ([$^{3}$H]SAM) into blocked methylated 5′ termini of newly synthesized RNA. Approximately 10% of the RNA chains initiated in vitro with [$\beta$-$^{32}$P]ATP are subsequently blocked by condensation of GMP to di- or triphosphate terminated RNA. The blocked termini can then be methylated by transfer of methyl groups from [$^{3}$H]SAM to the 7 position of the guanosine and 2′-O position of the adenosine to form m$^{7}$Gpp*pAm- capped terminus. In addition to conventional triphosphate caps, HeLa nuclear homogenates produce capping structures containing two phosphate residues in the pyrophosphate bridge. The two distinct cap forms were separated by DEAE-cellulose chromatography and analyzed. In contrast to triphosphate caps (m$^{7}$GpppXm) in which X can be any one of the four nucleosides (G, A, C, or U), in diphosphate caps (m$^{7}$GppXm), more than 95% of the penultimate nucleoside Xm is G. Incorporation of both [$\beta$-$^{32}$P]ATP and [$^{3}$H]SAM into caps was markedly reduced by low concentrations of $\alpha$-amanitin. However, an ammonium sulfate fraction of the nuclear homogenate can cap $\beta$-$^{32}$P-labeled RNA (pp*pA-RNA) to form m$^{7}$Gpp*pA-RNA, in the presence of 0.5 $\mu$g/mL of $\alpha$-amanitin. Therefore, the nuclear capping enzyme is resistant to this drug. Our results indicate that RNA polymerase II primary transcripts are the substrate for the cellular capping enzyme and that the $\beta$ phosphate in the pyrophosphate bridge (m$^{7}$G$^{\gamma}$p$^{\beta}$p$^{\alpha}$pXm) is derived from the 5′ ends of the RNA chains.

A wide variety of eukaryotic and viral mRNAs have been found to contain capping structures of the type m$^{7}$G(5′)ppp(5′)Xm at their 5′ termini (7-methylguanosine linked by 5′-5′ pyrophosphate bridge to the adjacent nucleotide) (for review, see Shatkin, 1976a). Capping structures are important for ribosome binding and mRNA translation (Muthukrishnan et al., 1975, 1976; Both et al., 1975a, 1976b) and cap analogues can cause inhibition of capped mRNA translation (Hickey et al., 1976; Canaani et al., 1976; Groner et al., 1976; Roman et al., 1976) as well as impairment of IF-M$_3$ mRNA interaction (Shafritz et al., 1976). It has also been suggested that capping and methylation may play a role during biogenesis of mammalian mRNAs and virus replicating in the nucleus (Rothman et al., 1974; Cory & Adams, 1975b; Salditt-Georgieff et al., 1976). The reactions involved in the formation of caps at the 5′ termini of viral mRNAs have been worked out for several viruses. It has been shown that vaccinia virus (Wei & Moss, 1975; Urishibara et al., 1975), reovirus (Furuichi et al., 1975), cytoplasmic polyhydrosis virus (cpv) (Furuichi & Miura, 1975), and vesicular stomatitis virus (VSV) (Abraham et al., 1975) particles contain virion-associated activities that are capable of methylating and capping these viral mRNAs. Three distinct enzymatic activities were implied (Furuichi et al., 1976; Moss et al., 1976) and subsequently isolated from vaccinia virus (Ensinger et al., 1975; Martin et al., 1975; Martin & Moss, 1975). In vaccinia virus, reovirus, and cpv, the capping reaction involves the transfer of guanosine monophosphate from GTP to the diphosphate terminated mRNA (ppX-) followed by methylation at the 7 position of the guanosine to form a m$^{7}$GpppX- terminus. However, a different mechanism that involves capping of a 5′-monophosphate end (pX-) was found for vesicular stomatitis virus (VSV) (Abraham et al., 1975).

Little is known about the biochemical steps and enzymes involved in the synthesis of capping structures in eukaryotic nuclear and messenger RNAs. It was previously reported that isolated HeLa nuclei (Groner & Hurwitz, 1975) and L cells nuclei (Winicov & Perry, 1976) incubated in vitro are able to form blocked methylated 5′ termini under conditions of RNA synthesis. $\alpha$-Amanitin, a specific inhibitor of RNA polymerase II, markedly reduced formation of blocked termini. Thus, there appears to be some relationship between RNA synthesis catalyzed by HeLa and L cells nuclei and incorporation of [$\alpha$-$^{32}$P]GTP (Groner & Hurwitz, 1975) or [$^{3}$H]SAM (Winicov & Perry, 1976) into 5′ caps. Recently, an enzyme that specifically methylates the guanosine residue of capping structures (RNA guanine-7-methyltransferase) was isolated from HeLa cells (Ensinger & Moss, 1976).

In order to gain further information about the mechanism of capping in eukaryotic cells, experiments were performed to

† From the Department of Virology, Weizmann Institute of Science, Rehovot, Israel. *Received August 19, 1977.* This research was supported by a grant from the United States-Israel Binational Science Foundation (BSF), Jerusalem, Israel, and in part by Contract NOI CP33220 from the National Cancer Institute.

0006-2960/78/0417-0977$01.00/0 