^{19}F Nuclear Magnetic Resonance Studies of Lipid Fatty Acyl Chain Order and Dynamics in *Acholeplasma laidlawii* B Membranes. Orientational Order in the Presence of Positional Isomers of *trans*-Octadecenoic Acid†

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ABSTRACT: The molecular order parameters, S_{mol}, of the lipid hydrocarbon chains of membranes of *Acholeplasma laidlawii* B, enriched with one of three positional isomers of *trans*-octadecenoic acid plus small amounts of one of a series of isomeric monofluoropalmitic acids, have been determined by using fluorine-19 nuclear magnetic resonance spectroscopy (^{19}F NMR). Orientational order profiles were similar in the liquid-crystalline state for each isomeric *trans*-octadecenoic acid and reflected a position-dependent, local disordering effect of trans unsaturation on the neighboring monofluoropalmitate chain. At temperatures below the lipid phase transition, overall orientational order increased profoundly, approaching the theoretical maximum at very low temperatures. A strong disordering effect of trans unsaturation was manifest as a head to tail gradient of order which remained in the gel state. The extent of disordering in the gel state was position dependent, being more pronounced for isomers with sites of trans unsaturation further from the carbonyl head group of the fatty acid. Differential scanning calorimetry studies indicated the possibility of head-group immiscibility in the polar lipid fraction from *A. laidlawii* membranes in the presence of *trans*-octadecenoic acids. Nevertheless, normalization of the values of S_{mol} with respect to the gel to liquid-crystalline phase transition demonstrated that the various isomeric *trans*-octadecenoic acids behaved as a class, achieving overall greater ordering than *cis*-octadecenoic acids but lesser ordering than straight-chain saturated fatty acids in the gel state.

The trans double bond, although not a common or usually even a natural constituent of biological membranes, has nevertheless been of interest in the experimental manipulation of membrane fatty acid composition. Fatty acids containing sites of trans unsaturation are capable of supporting the growth of unsaturated fatty acid auxotrophs of *Escherichia coli*, can be incorporated by *Mycoplasma capricolum* and *Acholeplasma laidlawii* to the extent that their membrane lipids are virtually homogeneous with respect to these fatty acids, and apparently are able to support normal membrane functioning in these circumstances [the effects of membrane lipids on growth and membrane activities have recently been reviewed by McElhaney (1982a)]. The trans double bond functions to "fluidize" the membrane, as do a variety of fatty acyl structural substituents, by lowering the temperature of the calorimetrically observable lipid phase transition [for a recent review, see McElhaney (1982b)].

Detailed insights into both the rates and amplitudes of the various hydrocarbon chain motions which cumulatively constitute membrane lipid fluidity can be obtained by using nuclear magnetic resonance (NMR)[1] techniques. Until recently, the range of fatty acyl structures studied via NMR has increased but slowly, being restricted primarily to studies of straight-chain saturated or cis-unsaturated systems due to the necessity, and the accompanying difficulty and expense, of synthesizing specifically enriched fatty acids of varying structure [for recent reviews, see Seelig & Selig (1980), Jacobs & Oldfield (1981), and Davis (1983)]. Thus, the manner in which many of the structural strategies employed in nature contrive to modulate membrane fatty acyl chain motions remains largely conjectural. However, the advent of ^{19}F NMR analysis of monofluoropalmitic acids (MFPA), as described in recent reports from this laboratory (McDonough et al., 1983; Macdonald et al., 1983, 1984, 1985), promises to permit a rapid, systematic survey across a wide range of diverse fatty acyl structures using NMR techniques.

Monofluoropalmitic acids are relatively nonperturbing, inexpensive, sensitive, and versatile membrane nuclear spin probes which, when biosynthetically incorporated into the membrane glycerolipids of, for example, the organism *A. laidlawii*, report a qualitative and quantitative picture of the physical state of membrane fatty acyl chains very similar to that obtained via ^{2}H NMR (McDonough et al., 1983; Macdonald et al., 1983, 1984). Moreover, the ^{19}F NMR spectra of MFPA in both the liquid-crystalline state and the gel state can be interpreted in terms of a single orientational order parameter, S_{mol} (Macdonald et al., 1984). Therefore, the amplitude of fatty acyl chain motions and, hence, the average chain configuration can be described in the gel state by using ^{19}F NMR, in addition to the normally accessible liquid-crystalline state. Furthermore, the wide range of chemical shifts undergone by the fluorine nucleus in the NMR experiment permits the simultaneous and independent monitoring of the physical state of both membrane protein and lipid when the two are both fluorine labeled (Dettman et al., 1982, 1984).

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[1] Abbreviations: NMR, nuclear magnetic resonance; DSC, differential scanning calorimetry; DTA, differential thermal analysis; FID, free induction decay; PC, phosphatidylcholine; MFPA, monofluoropalmitic acid(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; MGDG, monoglucosyl diglycoside; DGDG, diglucosyl diglyceride; PG, phosphatidylglycerol; DPPC, dipalmitoylphosphatidylcholine.

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In this report, we extend our studies of the orientational order of MFPA incorporated into the membrane lipids of *A. laidlawii* in the presence of fatty acids containing various structural and positional substituents to the cases of three positional isomers of *trans*-octadecenoic acid. We chose *A. laidlawii* because this organism readily incorporates exogenously supplied fatty acids, and when de novo fatty acid biosynthesis is inhibited with, for example, the protein avidin, its membrane lipids can be made virtually homogeneous with respect to a particular fatty acid (Silvius & McElhaney, 1978). Hence, the fatty acid composition and the membrane physical properties can be manipulated in a biological situation almost at will. In addition, this organism contains only a single membranous structure, the plasma membrane, so that the acquisition of relatively homogeneous membrane preparations is considerably simplified (Razin, 1975). Furthermore, previous ^{19}F NMR studies on *A. laidlawii* membranes enriched with a range of structurally diverse fatty acids will provide a basis for comparison with the results of the present study.

The ^{19}F NMR orientational order parameters of *A. laidlawii* membranes enriched with small amounts of one of a series of positional isomers of MFPA plus large amounts of one of the *trans*-octadecenoic acids, 18:1tΔ6, -Δ9, or -Δ11, have been obtained at temperatures corresponding to either the liquid-crystalline or the gel state. The resulting bilayer order profiles provided evidence of specific local effects of the trans double bond which could be correlated with the position of the site of unsaturation in either of the two phase states. Normalization of the orientational order data with respect to the calorimetrically determined gel to liquid-crystalline phase transitions then permitted a comparison of the overall temperature dependence of orientational order in the presence of trans unsaturation with that observed in the presence of other structural substituents. A coherent picture of the manner in which the trans-unsaturated species affects the bilayer stability emerged when these specific local and general overall effects were considered together.

Materials and Methods

Materials

The synthesis of the various monofluoropalmitic acids has been described elsewhere (McDonough et al., 1983). All other fatty acids used were products of NuChek Prep Co. (Elysian, MN). All solvents used were redistilled, and all chemicals used were of reagent grade or better.

Methods

Cell Culture, Membrane Isolation, Lipid Analysis, and Differential Scanning Calorimetry. The growth medium and conditions used for culturing *A. laidlawii* B with fatty acids and avidin have been described previously (Silvius & McElhaney, 1978). Membranes were prepared from late log-phase cultures of this organism by osmotic lysis essentially as described elsewhere (Silvius et al., 1977). The extraction and purification of total membrane polar lipids, the preparation of methyl esters, and the analysis by gas–liquid chromatography have been described before (Saito & McElhaney, 1977). Gel to liquid-crystalline phase transition endotherms of total membrane polar lipids were obtained by differential scanning calorimetry as previously described (Macdonald et al., 1984).

^{19}F NMR. Membrane samples for NMR analysis were suspended in buffer (0.154 M NaCl, 0.05 M Tris-HCl, and 20 mM β-mercaptoethanol, pH 7.4) diluted 20-fold with 95% deuterium oxide. ^{19}F NMR spectra were collected at 254.025 MHz on a Bruker HXS-270 NMR spectrometer equipped with a ^2H lock, operating in the Fourier transform mode, by using quadrature detection at a spectral width of $\pm$50 000 Hz. Bessel filters with a filter width of $\pm$100 000 Hz were employed. The temperature of the probe was maintained at the specified temperature to within $\pm$1 °C. Samples were equilibrated at a particular temperature for 30 min prior to data acquisition. Fluorine nuclei were subjected to a 15-μs ($\sim$75°) pulse followed by a 10-μs delay and a 20-ms acquisition time. The recycle time was 200 ms. Typically, 20K scans were accumulated for samples at which the temperature was above the gel to liquid-crystalline phase transition, and 100K scans were accumulated for samples at which the temperature was below the gel to liquid-crystalline phase transition. The first three points of the free induction decay (FID) were corrected for distortions associated with the receiver dead time by an extrapolation of the early portion of the FID back to time zero such that the signal intensity in the in-phase channel approximated a t^2 time dependency and that of the out-of-phase channel a t^1 dependency (Bloom et al., 1978). The signal to noise ratio was enhanced with an exponential multiplication which corresponded to a line broadening of 100 Hz, and the free induction decay was Fourier transformed to 2K data points in the real domain. The integrity of the spectrum and the flatness of the base line were taken as indicating that the extrapolation procedure had been performed correctly.

Results and Discussion

Fatty Acid Composition of A. laidlawii B Membranes. Tables I and II list the fatty acid composition for each case of supplementation with a particular combination of isomeric *trans*-octadecenoic acid plus isomeric monofluoropalmitic acid. The membrane fatty acid profiles were highly similar regardless of the position of the trans double bond or of the monofluoro substituent. The exogenously supplied fatty acids generally accounted for 90–95 mol % of the membrane fatty acids, with the products of de novo biosynthesis (12:0, 14:0, 16:0, and 18:0; Saito et al., 1977a) accounting for the remainder. The proportion of trans acid to monofluoro acid provided in the supplement was retained in the *A. laidlawii* membranes.

Thermotropic Behavior of A. laidlawii B Membrane Lipids. A valid comparison of the effects of a variety of fatty acyl structures on orientational order requires that the thermotropic effects of the structural variations be taken into account. Figure 1 illustrates the gel to liquid-crystalline phase transition endotherms obtained by DSC for the membrane lipids of *A. laidlawii* supplemented with 85% 18:1tΔ9 plus 15% monofluoro-16:0 for each particular monofluoropalmitic acid employed. These phase transition endotherms are quite distinct from those reported previously from this laboratory for *A. laidlawii* membrane lipids highly enriched with particular fatty acids [e.g., see McDonough et al. (1983), Macdonald et al. (1984), and Macdonald et al. (1985)] in that the presence of two separate thermotropic events could be discerned. The lower temperature transition (T_1) accounted for 60–65% of the total transition enthalpy while the higher temperature transition (T_2) accounted for 35–40% of the total enthalpy. The separation of the two transitions ($\Delta T_1 - T_2$) was largest when the monofluoro substituent was nearer the carbonyl head group or the methyl terminus of the acyl chain and smallest when the monofluoro substituent was located near the center of the acyl chain. These characteristics, the presence of two separate thermotropic events and their relative enthalpies and the dependence of their separation on the position of the monofluoro substituent, were observed for each of the three isomeric *trans*-octadecenoic acids employed.

Table I: Composition of *A. laidlawii* Membranes Enriched with *trans*-Octadecenoic Acids plus Monofluoropalmitic Acids

supplement[a]	mol % fatty acid					
	12:0	14:0	16:0	18:1	*x*F-16:0	other
15% 6F-16:0 + 85% 18:1tΔ6	0.79	1.56	2.03	80.01	11.06	4.54
8F-16:0	0.53	0.25	0.37	83.05	12.42	3.37
10F-16:0	0.27	0.19	0.41	80.36	9.31	9.46
12F-16:0	0.33	0.37	1.50	79.90	9.61	8.29
14F-16:0	0.94	0.48	1.76	80.11	10.18	6.52
15% 6F-16:0 + 85% 18:1tΔ9	0.57	0.18	5.27	82.50	9.50	
8F-16:0	0.49	0.16	5.12	83.23	11.00	
10F-16:0	0.42	0.13	5.13	82.16	12.16	
12F-16:0	0.54	0.30	6.21	78.32	7.33	7.30
14F-16:0	1.27	0.54	5.73	78.99	7.22	5.25
15% 6F-16:0 + 85% 18:1tΔ11	0.32	0.43	2.23	82.89	14.13	
8F-16:0	0.37	0.19	0.42	83.31	14.31	1.39
10F-16:0	0.25	0.22	0.22	84.23	14.67	0.42
12F-16:0	0.34	0.27	0.47	83.95	14.20	0.77
14F-16:0	0.52	0.37	0.41	84.40	14.01	0.29

[a] Final fatty acid concentration was 0.12 mM.

Table II: Composition of *A. laidlawii* Membranes Enriched with Various Proportions of Elaidic Acid plus Palmitic Acid

supplement[a]	mol % fatty acid			
	12:0	14:0	16:0	18:1
100% 18:1tΔ9	0.4	0.5	0.9	98.2
90% 18:1tΔ9 + 10% 16:0	0.3	0.2	11.3	88.2
80% 18:1tΔ9 + 20% 16:0	0.3	0.1	20.0	79.6
70% 18:1tΔ9 + 30% 16:0	0.3	0.1	30.7	68.9
60% 18:1tΔ9 + 40% 16:0	0.4	0.1	39.6	59.9
50% 18:1tΔ9 + 50% 16:0	0.3	0.1	49.8	49.8

[a] Final fatty acid concentration was 0.12 mM.

Tables III and IV summarize the calorimetric data for these membranes and include, for comparison, data previously reported by Silvius et al. (1980) for *A. laidlawii* membranes and phosphatidylcholines (Silvius et al., 1979) containing *trans*-octadecenoic acids. The P2/P1 ratio (defined as the preference of a particular fatty acid for esterification to the *sn*-2 vs. the *sn*-1 position, relative to palmitic acid) for 18:1tΔ9 reported by Saito et al. (1977b) suggests that *trans*-octadecenoic acids are 6 times as likely as palmitic acid to be esterified at the *sn*-2 position of the glycerol backbone of *A.* laidlawii membrane lipids. This preference is sufficient to suggest that when the supplement is 85% 18:1tΔ*x* plus 15% *y*F-16:0, the membrane lipids contain approximately 30% of a mixed acid species (*y*F-16:0/18:1tΔ*x*) and 70% of a diacid species (18:1tΔ*x*/18:1tΔ*x*). Since the lower temperature transition (T_1) observed here most closely corresponds to previously reported

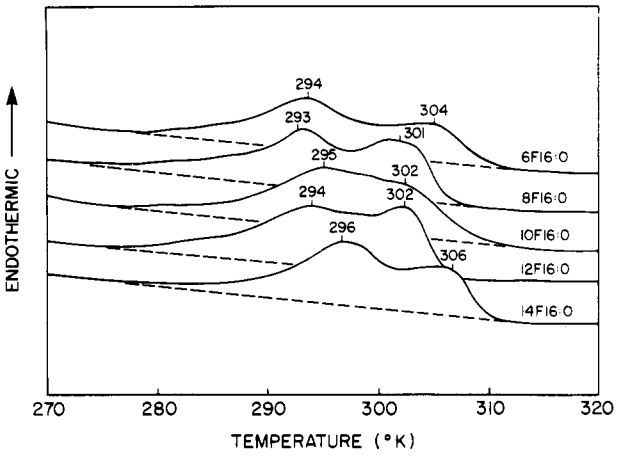

FIGURE 1: DSC endotherms of membrane lipids of *A. laidlawii* supplemented with 85% 18:1tΔ9 plus 15% of various monofluoropalmitic acids. The scan rate was 5 K/min. (---) Interpolated base line.

Table III: Summary of Calorimetric Data on Membrane Lipids from *A. laidlawii* Grown in the Presence of *trans*-Octadecenoic Acids plus Monofluoropalmitic Acids

supplement	temp (K)[a]					% total[b]	
	T_1	T_2	T_m	$\Delta T_1 - T_2$	ΔT_{10-90}	ΔH_1	ΔH_2
15% 6F-16:0 + 85% 18:1tΔ6	302	313	308	11	16	62	38
8F-16:0	303	309	306	6	11	59	41
10F-16:0	303	310	305	7	11	67	33
12F-16:0	301	313	305	12	15	65	35
14F-16:0	300	311	303	11	14	69	31
	302[c]	311[c]	305[c]	9[c]	13[c]	64[c]	36[c]
15% 6F-16:0 + 85% 18:1tΔ9	294	304	296	10	19	67	33
8F-16:0	293	301	295	8	18	59	41
10F-16:0	295	302	297	7	17	66	34
12F-16:0	294	302	296	8	16	67	33
14F-16:0	296	306	398	10	17	66	34
	294[c]	303[c]	396[c]	9[c]	17[c]	65[c]	35[c]
15% 6F-16:0 + 85% 18:1tΔ9	297	306	302	9	15	50	50
8F-16:0	299	308	302	9	22	65	35
10F-16:0	301	303	302	2		15	
12F-16:0	301	307	303	6	12	59	41
14F-16:0	302	308	303	6	6	60	40
	300[c]	307[c]	302[c]	7[c]	13[c]	58[c]	42[c]

[a] T_1 and T_2 correspond to the temperatures of the lower and higher temperature transitions, respectively, T_m equals the temperature at which the transition was 50% complete, $\Delta T_1 - T_2$ equals the separation of T_1 and T_2, and ΔT_{10-90} equals the temperature range over which the transition passes from 10% to 90% of completion. [b] The total area under the transition endotherm was used as 100%. ΔH_1 or ΔH_2 equalled the area below or above the temperature minimum between T_1 and T_2. [c] Mean values.

Table IV: Comparison of Mean Transition Temperatures (K) with Previously Reported Calorimetric Data

	present report			
fatty acid	T_1	T_2	*A. laidlawii*[a] T_c	PC[b] T_c
18:1tΔ6	302	311	295.2	
18:1tΔ9	295	304	293.1	12.9
18:1tΔ11	300	306	293.0	13.2

[a] Data from Silvius et al. (1980). [b] Data from Silvius & McElhaney (1979).

values of the phase transition temperatures of *A. laidlawii* membranes or phosphatidylcholines containing *trans*-octadecenoic acids, it would seem reasonable to suggest that this transition could be attributed to a diacid species while the higher temperature transition (T_2) could result from the presence of a somewhat immiscible mixed-acid species containing a generally higher melting monofluoropalmitic acid. This suggestion is further supported by the observation that the proportion of the enthalpies of the two transitions, $\Delta H_1/\Delta H_2$, was generally 65/35, roughly corresponding to the expected proportions of the diacid/mixed-acid species under these conditions.

However, as reasonable as these arguments might seem, the possibility that head-group immiscibility as well as acyl chain immiscibility contributed to the observed phase separations cannot be excluded. DSC endotherms of *A. laidlawii* lipids are generally somewhat asymmetric (McElhaney, 1982b), and the individual head-group species in *A. laidlawii* membranes, when isolated, exhibit significantly different and sometimes complex phase behavior (Silvius et al., 1980), suggesting that the asymmetric transitions observed with total *A. laidlawii* lipid extracts may be composites of unresolved individual glycerolipid species transitions. It is noteworthy as well that the differential thermal analysis (DTA) endotherms for *A. laidlawii* membrane lipids highly enriched with 18:1tΔ9, reported by McElhaney (1974), show the presence of two distinct thermotropic events. Moreover, it is generally held that immiscibility originating in the fatty acyl chains can be correlated with the differences in melting temperatures of the species involved (Mabrey & Sturtevant, 1976). It does not seem a priori that the melting temperatures of a di-18:1tΔ9 species and a mixed 16:0/18:1tΔ9 species should be sufficiently different as to lead to the extensive phase separation observed here.

In an attempt to distinguish the effects of head-group vs. acyl chain immiscibility, we obtained the DSC endotherms at a number of scan rates (10, 5, 2, and 1 °C/min) of membrane lipids of *A. laidlawii* containing various ratios of 16:0 to 18:1tΔ9. These endotherms are illustrated in Figure 2 for scan rates of 5 °C/min. At all proportions of 16:0 tested (0, 10, 20, 30, 40, and 50 mol % 16:0 in 18:1tΔ9), two separate thermotropic transitions could be discerned. With increasing amounts of 16:0, three occurrences were noted. First, the temperatures of both the lower temperature (T_1) and higher temperature (T_2) transition increased, by approximately 7 and 14 °C, respectively, as the concentration of the higher melting palmitate was increased over the range 0–50 mol %. Second, the separation of the two transitions, $\Delta T_1 - T_2$, progressively increased from about 4 °C at 100 mol % 18:1tΔ9 to almost 11 °C at 50 mol % 16:0. Third, the proportion of the total transition enthalpy attributable to the higher temperature transition, T_2, decreased from approximately 50% at the lowest concentration of 16:0 to less than 30% at the highest concentrations of 16:0. These occurrences were independent of the scan rate at which the endotherms were obtained, indicating that there were no kinetic limitations on the rates of

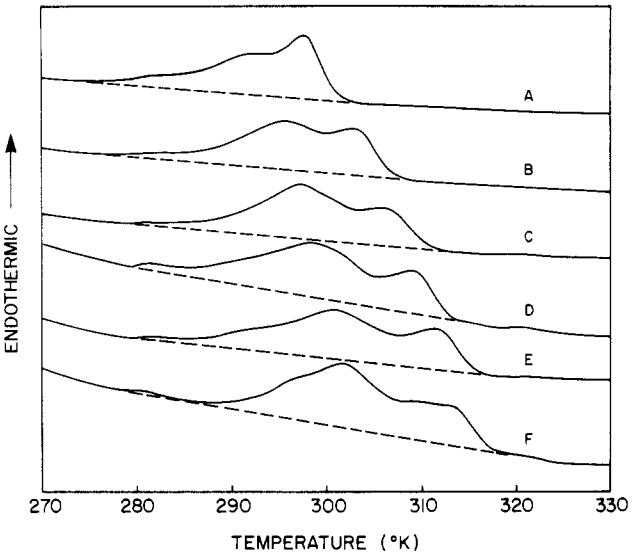

FIGURE 2: DSC endotherms of membrane lipids of *A. laidlawii* supplemented with various proportions of 18:1tΔ9 plus 16:0 with a scan rate of 5 K/min. (---) Interpolated base line. (A) 100% 18:1tΔ9; (B) 90% 18:1tΔ9 + 10% 16:0; (C) 80% 18:1tΔ9 + 20% 16:0; (D) 70% 18:1tΔ9 + 30% 16:0; (E) 60% 18:1tΔ9 + 40% 16:0; (F) 50% 18:1tΔ9 + 50% 16:0.

the phase transitions which might obscure the true phase behavior, although the temperatures of both the lower and higher temperature transitions increased by approximately 5 °C from the lowest to the highest scan rate.

Consider first the possible distribution of the two fatty acyl species 16:0 and 18:1tΔ9 among the major lipid classes of this organism. Saito & McElhaney (1977) observed that, although differences in the fatty acid composition of the glycolipid vs. the phospholipid membrane components could be discerned when endogenous fatty acid biosynthesis was predominant, under conditions of high enrichment with exogenous fatty acids these differences disappeared. Thus, we would expect that, at each ratio of 16:0/18:1tΔ9 tested, the fatty acid composition of each of the major membrane polar lipids of *A. laidlawii* would be approximately identical and would reflect the ratio of 16:0/18:1tΔ9 provided exogenously. Furthermore, the P2/P1 ratio of 18:1tΔ9 relative to 16:0 reported by Saito et al. (1977b) was approximately 6 for each monoglucosyl diglyceride (MGDG), diglucosyl diglyceride (DGDG), and phosphatidylglycerol (PG) and was practically independent of the proportion of the two fatty acids compared. We would further expect, therefore, that at each ratio of 16:0/18:1tΔ9 and for each of the major polar lipid components, the palmitic acid chains will be preferentially esterified to the *sn*-1 position and that the proportion of mixed-chain (16:0, 18:1tΔ9) and diacid (18:1tΔ9, 18:1tΔ9) species will vary directly as the ratio of 16:0/18:1tΔ9 provided exogenously, from 100% diacid species at 100% 18:1tΔ9 to 100% mixed-acid species at 50/50 16:0/18:1tΔ9. These various points indicate that a priori one would expect little difference in the fatty acid composition or positional distribution of fatty acids within any of the major polar lipid components of *A. laidlawii* under these experimental conditions.

Consider next the expected changes in the polar lipid composition as the ratio of 16:0/18:1tΔ9 is varied. The ratio (MGDG + DGDG)/PG has been reported to be relatively invariant with altered fatty acid composition, generally averaging about a value of 3 (Silvius et al., 1980). In contrast, the ratio MGDG/DGDG was found to be highly variable, equalling 0.86 at high levels of 18:1tΔ9 and increasing markedly as the degree of saturation was increased (Silvius

et al., 1980). Therefore, we expect the neutral lipid composition to change from predominantly DGDG at 100% 18:1tΔ9 to predominantly MGDG at 50% 16:0/50% 18:1tΔ9. It can further be noted that the neutral lipids in general and DGDG in particular display complex phase behavior in DTA studies of isolated lipids and melt at temperatures up to 10 °C higher than the overall membrane lipid phase transition (Silvius et al., 1980).

Turning then to the DSC endotherms we obtained with different ratios of 16:0/18:1tΔ9, we observe effective phase separation at all proportions. Assuming 100% diacid species at 100% 18:1tΔ9 and 100% mixed-acid species at 50% 16:0/50% 18:1tΔ9, it must be concluded that head-group immiscibility is responsible for the observed phase separations under these conditions since little fatty acid compositional heterogeneity should be expected. Since the temperature of both the lower temperature and higher temperature transitions increased as the proportion of 16:0 was increased, it is apparent that this fatty acid was readily incorporated into the polar lipid species responsible for both transitions. Since the enthalpy of the higher temperature transition decreased relative to that of the lower temperature transition as the proportion of 16:0 increased, while it is expected that the ratio of MGDG/DGDG should increase greatly under the same conditions, it is not unreasonable to conclude that the higher temperature transition is attributable to the immiscibility of the generally higher melting, bulky DGDG head-group species. This conclusion further suggests that head-group immiscibility is responsible for the thermotropic behavior of membrane lipids containing 85% 18:1tΔ*x* plus 15% *y*F-16:0. Thus, the monofluoropalmitic acids are probably not particularly enriched in the polar lipid species responsible for either the lower or the higher temperature transition but are most likely evenly distributed among all species, as concluded previously from direct experimentation (McDonough et al., 1983). As a basis for comparison among the various isomeric *trans*-octadecenoic acids studied here, we have therefore used the center of the entire phase transition rather than one or the other of the two distinct thermotropic events.

^{19}F NMR Analysis of A. laidlawii B Membranes. Examples of experimentally obtained ^{19}F NMR spectra of *A. laidlawii* B membrane samples as a function of temperature, and the corresponding computer-generated simulated spectra, are illustrated in Figure 3 for the case of enrichment with 15 mol % 6F-16:0 plus 85 mol % 18:1tΔ9. At a temperature above the lipid phase transition, the ^{19}F NMR spectrum consisted of a single, narrow, somewhat asymmetric resonance line reflecting the extensive and rapid fatty acyl chain motions characteristic of the liquid-crystalline state. With decreasing temperature and altered lipid phase state, the spectrum progressively broadened in response to the increasingly restricted chain motions accompanying the transition to the gel state. An essentially superlorentzian line shape was observed at all temperatures.

It is generally accepted that, in the region of the phase transition, domains of gel-state and liquid-crystalline-state lipid coexist and that the exchange of lipid molecules between these domains is slow on the NMR time scale. Thus, the NMR spectrum in the coexistence region can be considered to be a superposition of gel-state and liquid-crystalline-state components, and their individual contributions are readily discernible in ^{13}C [e.g., see Blume et al. (1982)] or ^{2}H NMR [e.g., see Jarrell et al. (1981)] spectra. The ^{19}F NMR spectrum at 289 K should also be a superposition of gel-state and liquid-crystalline-state components since this temperature is within

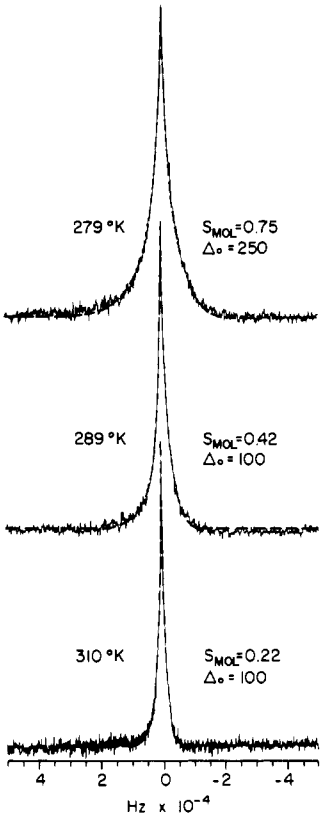

FIGURE 3: Typical ^{19}F NMR experimental and corresponding simulated spectra. Spectra of *A. laidlawii* membranes enriched with 85% 18:1tΔ9 plus 15% 6F-16:0 are shown. Parameters of fit are indicated beside simulated spectra: Δ_o = intermolecular dipole interaction; S_{mol} = molecular order parameter.

the lipid phase transition. It is pertinent to note that ^{19}F NMR spectra obtained in the coexistence region can be simulated by the addition of 100% fluid spectra and 100% gel spectra in the proper proportion and that these simulated spectra are indistinguishable from experimentally obtained spectra (Macdonald, 1984). Therefore, although it is not readily apparent from a visual examination, the ^{19}F NMR spectrum in the coexistence region can be considered a superposition of gel-state and liquid-crystalline-state components. The dipolar broadening which influences the fluorine line shape may obscure that region of the spectrum where the contribution of individual components might be most readily discernible. The results of Blume et al. (1982) indicate that the ^{13}C NMR spectra in the coexistence region are not a *simple* superposition of fluid and gel components but rather that each component is subject to exchange broadening, this effect being more obviously manifest by the fluid component. Moreover, the apparent exchange rate increased as the cooperativity of the lipid phase transition decreased. Thus, the broad, less cooperative (relative to synthetic model membranes) lipid phase transitions observed in *A. laidlawii* B may influence the exchange rate and act to somewhat obscure the contribution of individual fluid and gel components to the ^{19}F NMR spectrum. Furthermore, the character of the individual spectra components, fluid and gel, will alter with temperature, the fluid component broadening and the gel component narrowing relative to their breadth in the purely liquid-crystalline or purely gel state. Each of these considerations could conspire, individually or collectively, to obscure the contribution of distinct gel phase and fluid phase components to the ^{19}F NMR spectrum in the coexistence region.

The ^{19}F NMR line shape is influenced by both chemical shift anisotropic and dipole–dipole interactions which are

modulated by the anisotropic motions peculiar to a lipid molecule embedded in a bilayer membrane. The superposition quality of these two interactions and their relative magnitudes and the axially symmetric molecular motions in the lipid bilayer lead to the superlorentzian line shapes shown in Figure 3. As such, there is no one simple spectral parameter which is unequivocally related to the orientational order of the bilayer lipids, and we have therefore resorted to spectral simulations in order to extract this information.

In practice, the maximum chemical shift anisotropy and the maximum F–H intrachain dipolar interaction were first estimated for a monofluoropalmitate-containing lipid whose fatty acyl chains had assumed an all-trans configuration and which was experiencing rapid (on the ^{19}F NMR time scale) rotations about the lipid long axis. The decrease in the observed chemical shift anisotropy and intrachain dipolar interaction from their estimated maximum was quantitated by the introduction of an orientational order parameter, S_{mol}. The hydrocarbon chain orientational order parameter, S_{mol}, quantitates the amplitude of the time-averaged angular excursions of the fatty acyl chains away from an axis perpendicular to the plane of the bilayer. Tilting of the whole molecule with respect to the bilayer normal, as well as trans–gauche isomerization at individual methylene segments, can contribute to the deviation of S_{mol} from a value of 1.0, indicative of acyl chains assuming a fully extended all-trans configuration and aligned parallel to the bilayer normal, toward a value of 0, at which individual chain segments would experience essentially free isotropic motion. Seelig (1977) defined the molecular order parameter such that

$$S_{mol} = \tfrac{1}{2}\langle 3\cos^2\theta - 1\rangle$$

Here, θ is the angle between the segment direction and the bilayer normal where the segment direction is defined as the normal to the plane formed by the H–C–H atoms of the methylene segment. The broken brackets represent an average over time.

The mathematical model employed to generate the spectral simulations has been described in detail elsewhere (Macdonald et al., 1983, 1984). The validity of the model and, hence, of the extracted values of S_{mol} rests upon the estimated values of the maximum chemical shift anisotropy and intrachain dipolar interaction as well as the assumption of effectively axially symmetric motions of the lipid molecules.

The value of the maximum chemical shift anisotropy was estimated from a consideration of the fluorine chemical shift tensor elements reported for Teflon (Mehring, 1971) and was found to be 82.2 ppm (Macdonald et al., 1983). If this value were an over- or underestimate, the extracted values of S_{mol} would be correspondingly under- or overestimated. In the absence of a direct measurement of the chemical shift tensor elements of a monofluoro-type compound, we have consistently used the above estimated value as the maximum. Although it is possible that the values of the fluorine chemical shift tensor elements or the orientation of the tensor principal axis system might alter under different experimental conditions, it is most likely that these quantities are insensitive to the changes in temperature, label position, etc. to which we have subjected them. Thus, the alterations in the ^{19}F spectra have been attributed entirely to changes in the value of S_{mol}.

The maximum F–H intrachain dipolar interaction was determined to be approximately 20 000 Hz from a study of the magnetic field strength dependence of the ^{19}F spectrum of a monofluoropalmitate incorporated into a lipid bilayer (Macdonald et al., 1983), and this value was in good agreement with that estimated theoretically from a consideration of the geometry of a monofluoromethylene segment within an acyl chain (Macdonald, 1984).

Of paramount importance to our spectral simulations is the assumption of effectively axially symmetric motions of the bilayer lipid molecules. In the absence of long-axis rotational motions of the whole lipid or torsional motions of the fatty acyl chains of sufficient rapidity on the ^{19}F NMR time scale to provide effective axial symmetry, it would no longer be possible to completely describe the orientational order of the monofluoromethylene segment in terms of a single order parameter. It is clear that the ^{2}H NMR spectrum reflects axially symmetric lipid motions in the liquid-crystalline state but not in the gel state [e.g., see Seelig (1977)]. On the other hand, ^{13}C NMR spectra are characteristic of axial symmetry even to temperatures 40 °C below the lipid phase transition [e.g., see Wittebort et al. (1981)], and this difference between the gel-state ^{2}H and ^{13}C spectra can be attributed to the approximately 1 order of magnitude difference in the time scales relevant to the two procedures (Davis, 1983). The time scale relevant to the ^{19}F spectrum more closely corresponds to that of ^{13}C (Macdonald et al., 1984) so that on theoretical grounds one would expect effective axial symmetry to be manifest in both the gel-state and liquid-crystalline-state ^{19}F spectra. The ^{19}F spectrum in the absence of axial symmetry has a broad Gaussian line shape in contrast to the superlorentzian obtained at 279 K (in the gel state) in Figure 3 (Macdonald et al., 1984). Furthermore, it is apparent that the spectral simulations, which explicitly assume axial symmetry, more than adequately reproduce the experimental ^{19}F line shapes in both the gel and the liquid-crystalline phase. Although these various points do not prove that on the ^{19}F NMR time scale axial symmetry exists in the gel phase, and it has not been demonstrated directly that the ^{19}F spectrum has an asymmetry parameter of zero (i.e., effective axial symmetry) or that it is necessarily possible to distinguish the presence of a nonzero asymmetry parameter (i.e., axial asymmetry), the weight of the present evidence, the theoretical considerations, the superlorentzian character of the gel-state ^{19}F spectra, and the successful simulations assuming an asymmetry parameter of zero clearly suggest that the motions of the lipid molecules in the gel state are of a nature and of sufficient rate to validate the assumption of axial symmetry on the ^{19}F NMR time scale.

Since the ^{19}F spectra in the region of the lipid phase transition can be considered a superposition of gel-state and liquid-crystalline-state components and since the ^{19}F spectrum in the coexistence region can apparently be adequately described in terms of a single order parameter (see Figure 3), the value of S_{mol} obtained at a temperature within the lipid phase transition must be considered an average of the orientational order of the fluid and gel components. This average value will be a function of the relative proportions of the two components as well as of the temperature dependence of the orientational order within any one gel or fluid domain over the temperature range of the phase transition.

The order profile of a lipid bilayer represents the variation of the order parameter S_{mol} with the position of the segment in the chain. ^{19}F NMR order profiles are illustrated in Figure 4 for each of the three isomeric *trans*-octadecenoic acids studied at temperatures above (310 K), just below (289 K), and well below (279 K) the gel to liquid-crystalline phase transition. The qualitative and quantitative similarities between the order profiles obtained by ^{19}F NMR and ^{2}H NMR have been described previously (Macdonald et al., 1983, 1984), and the two techniques appear to provide a very similar view of the configurations assumed by acyl chains in a lipid bilayer.

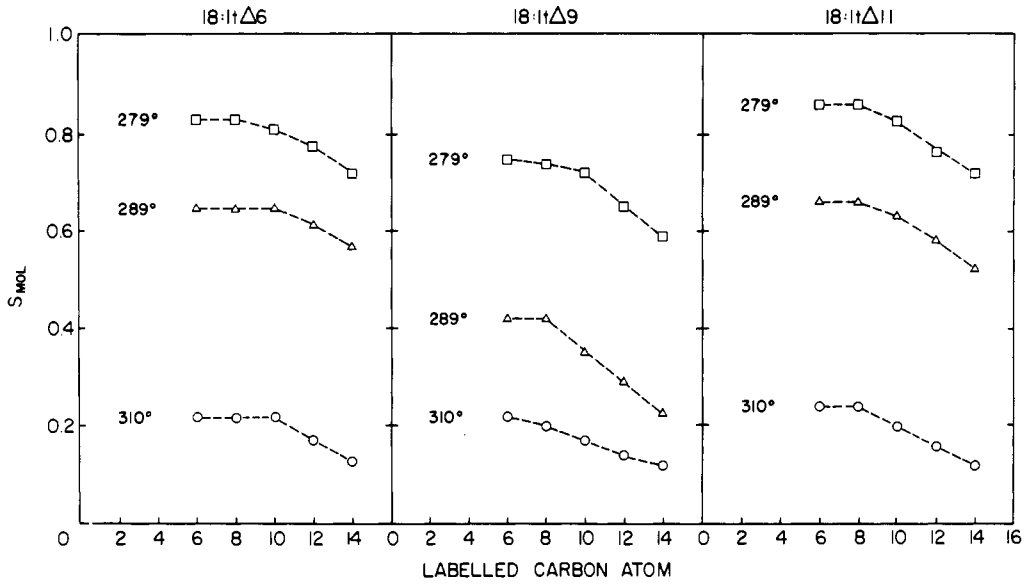

FIGURE 4: ^{19}F NMR order profiles of *A. laidlawii* membranes enriched with various isomers of *trans*-octadecenoic acid plus monofluorinated palmitic acids acquired at temperatures above (310 K), just below (289 K), and well below (279 K) the lipid phase transition.

In the liquid-crystalline state at 310 K, the order profiles were qualitatively similar for each isomeric *trans*-octadecenoic acid, with a region of relatively constant order preceding a progressive decline in order toward the methyl terminus of the acyl chain. Nevertheless, it was possible to observe a local disordering effect of the trans double bond in the ^{19}F NMR order profile as reported previously (Macdonald et al., 1983). The order profile of 18:1tΔ6 most closely resembled that of a straight-chain saturated fatty acid such as 15:0 or 16:0. Given the inequality of the penetration into the bilayer of the *sn*-1 and *sn*-2 esterified acyl chains and the fact that the nearest position to the carbonyl head group which was monitored was with 6F-16:0, this result is not surprising. Furthermore, it is expected that chain packing densities should increase substantially nearer the lipid polar head group so that local ordering or disordering effects of particular substituents should be less readily manifest. The local disordering effect of the trans double bond was most apparent in the case of 18:1tΔ9 but was also apparent in the order profile obtained in the presence of 18:1tΔ11. Seelig & Waespe-Šarčevič (1978) described the ^{2}H NMR order profile of 1-16:0,2-18:1tΔ9-PC and found no differences between the order profile of the elaidoyl chain and that of a palmitoyl chain in DPPC. However, the same may be said of the cis double bond after correction for geometric considerations despite its local ordering effect on a neighboring chain (Seelig & Seelig, 1977). Thus, the effect of a structural substituent on a neighboring chain such as observed by ^{19}F NMR can provide insights into the effects of particular substituents. It should be emphasized that these local effects observed by ^{19}F NMR are small in relation to the temperature dependence of segmental order even in the absence of a phase change. For example, Macdonald et al. (1985) noted that the ^{19}F NMR order profiles obtained in the presence of a series of isomers of *cis*-octadecenoic acid were very similar, regardless of the position of the site of cis unsaturation, provided they were obtained in the liquid-crystalline state.

At a temperature just below the phase transition (289 K), orientational order increased substantially at all positions for all isomeric *trans*-octadecenoic acids. Despite the predominance of gel-state lipid at this temperature, the values of the order parameters had not yet approached the theoretical maximum of 1.0. In addition, a head to tail gradient in orientational order was still apparent at 289 K. The much greater disorder of the 18:1tΔ9 isomer at 289 K may be simply due to the fact that at this temperature membranes enriched with this isomer were closer to their phase transition than either of the other isomers (see Tables III and IV).

At 279 K, each of these membrane preparations was well below its respective gel to liquid-crystalline phase transition. The order profiles at 279 K were characteristic of a highly ordered lipid state with individual values of S_{mol} approaching but not achieving the theoretical maximum. Despite this uniformly high ordering, the order profiles indicated that, even far below the phase transition, in the presence of a trans double bond a head to tail gradient of orientational order still existed. In keeping with the previously reported conclusion that certain structural substituents more readily manifest a disordering effect in the gel state when they are located further from the carbonyl head group of the fatty acid (Macdonald et al., 1985), it could be discerned that the head to tail gradient of order was more pronounced in the presence of 18:1tΔ9 or -Δ11 than in the presence of 18:1tΔ6. In fact, the gradient of order in the presence of 18:1tΔ11 was greater than that of 18:1tΔ6 even at 289 K. Thus, the trans double bond was apparently able to somewhat disrupt or prevent the assumption of a uniformly highly ordered state. This effect was therefore manifest as a gradient of disorder that remained in the gel state. It is pertinent to note that the gel-state order profiles of straight-chain saturated fatty acids obtained by ^{2}H NMR (Allegrini et al., 1983) or ^{19}F NMR (Macdonald et al., 1984) are relatively flat with very little head to tail decrease in orientational order. This disruption due to a trans double bond is, however, much less pronounced than that observed in the gel state in the presence of a cis double bond (Macdonald et al., 1985). The implication is then that the decrease in the temperature of the phase transition in the presence of a cis or trans double bond may be qualitatively correlated with the extent of disordering in the gel state attributable to a particular substituent.

In order to permit a direct comparison of the orientational order parameters obtained under a variety of physical conditions and in the presence of diverse structural substituents, it is necessary to refer to some common coinciding state. The gel to liquid-crystalline phase transition is certainly the single greatest effector of orientational order in membrane lipids, and its effects must be accounted for. In order to minimize

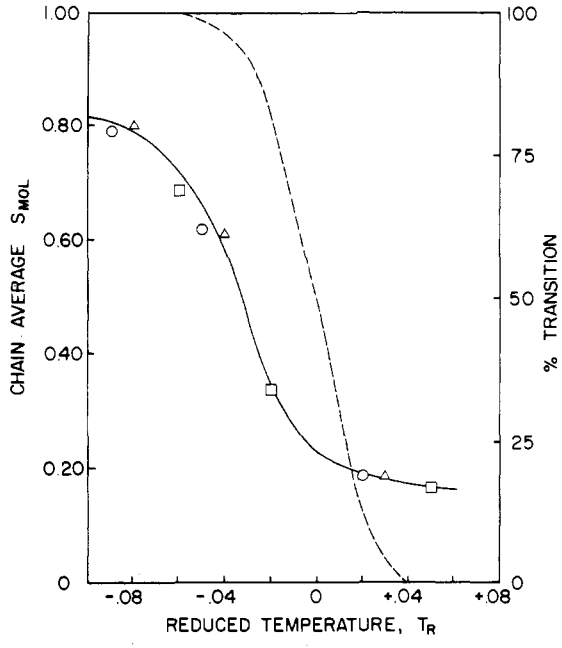

FIGURE 5: Normalization of the chain-average order parameter with respect to the particular phase transition temperature for each case of enrichment with a particular isomer of *trans*-octadecenoic acid. Chain-average order was calculated as the average of the five values of S_{mol} obtained at any one temperature for any one isomeric *trans*-octadecenoic acid. The mean value of T_m from Table III was used to calculate T_r. The dashed line shows the percent transition completed as a function of the reduced temperature. (○) 18:1tΔ6; (□) 18:1tΔ9; (△) 18:1tΔ11.

effects arising from differences in the thermotropic behavior of various fatty acid structures, it is common practice to normalize the order parameters with respect to the phase transition and refer to some reduced temperature, T_r, such that

$$T_r = (T - T_m)/T_m$$

where T_m is the phase transition temperature and T is the temperature of measurement in degrees kelvin (Seelig & Browning, 1978). Figure 5 compares the chain-average order parameters for each of the three isomers of *trans*-octadecenoic acid at the reduced temperature T_r. The chain-average order was simply the numerical average of the five values of S_{mol} obtained for each trans isomer at each temperature. The value of T_r was calculated by using the temperatures corresponding to the middle of the entire phase transition listed in Table III as T_m. When this comparison was made, it became apparent that the *trans*-octadecenoic acids behaved very similarly as a class with respect to the phase transition. In the liquid-crystalline state, overall order increased in an approximately linear fashion with increasing proximity to the phase transition. Once the center of the phase transition was traversed, overall order increased rapidly but only approached maximal values when the phase transition was greater than 90% complete. When an analogous comparison was made of the overall order of an isomeric series of *cis*-octadecenoic acids at a reduced temperature, the dependence of order on the phase transition was nearly identical with that observed with isomeric *trans*-octadecenoic acids in Figure 5 (Macdonald et al., 1985). Thus, the lipid phase transition appears to be the preeminent effector of overall order independent of the configuration of the double bond (cis or trans) or its location along the acyl chain.

CONCLUSIONS

The effects of the trans double bond, relative to a straight-chain saturated acyl chain, appear to be intermediate to the effects of the cis double bond in a number of senses. The decrease in the phase transition temperature of phospholipids resulting from the inclusion of a trans double bond, although pronounced, is far less than that which occurs upon substituting a cis double bond [e.g., see McElhaney (1982b)]. The marked dependence of transition temperature upon the position of the site of unsaturation in the presence of a cis double bond (Barton & Gunstone, 1975) is practically nonexistent in the presence of a trans double bond (Silvius & McElhaney, 1979). Monolayer studies indicate that phospholipids containing trans double bonds occupy cross-sectional areas intermediate to relatively expanded cis double bond containing and relatively condensed straight-chain saturated-containing phospholipids when compared at identical surface pressures (Chapman et al., 1966).

The trans double bond disrupts the gel-state orientational order of monofluoropalmitic acids in membranes of *A. laidlawii* to an extent that is intermediate to the effects of a cis double bond while demonstrating relatively less dependence upon the position of the site of unsaturation. These properties are consistent with at least a qualitative correlation between overall orientational order in the gel state and the temperature of the corresponding gel to liquid-crystalline phase transition. The dependence of orientational order on the proximity to the phase transition in both cis- and trans-unsaturated fatty acids demonstrates that, despite specific effects attributable to either substituent, the lipid phase transition is the largest effector of lipid orientational order.

Registry No. 18:1tΔ6, 7206-22-6; 18:1tΔ9, 7206-25-9; 18:1tΔ7, 7206-23-7; 16:0, 57-10-3.

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Studies on Transcription of 3′-Extended Templates by Mammalian RNA Polymerase II. Parameters That Affect the Initiation and Elongation Reactions†

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ABSTRACT: Addition of short sequences of dCMP residues to the 3′-OH end of duplex linear DNAs allows rapid and efficient transcription to be initiated at these sites by purified mammalian RNA polymerase II [Kadesch, T. R., & Chamberlin, M. J. (1982) *J. Biol. Chem. 257*, 5286–5295]. The use of such tailed DNA templates should allow biochemical studies on transcription elongation and termination with almost any desired DNA sequence. However, in vitro transcription with RNA polymerase II is aberrant in that the DNA template is not re-formed after transcription; rather, the DNA strands are separated, and most of the RNA product is found as a DNA–RNA hybrid. To better understand the factors that affect the process of transcription with these tailed DNA templates, we have varied a number of parameters that might be expected to play a role in the reaction. RNA polymerase II preparations from calf thymus, HeLa cells, and *Drosophila* all fail to displace the product RNA. However, RNA polymerase II from wheat germ gives only free RNA as a product, as does the *Escherichia coli* RNA polymerase. Hence, the displacement of the nascent RNA from a transcription complex seems to depend on some intrinsic property of the polymerase itself and not simply on the nature of the template. Variation of reaction conditions, or of the divalent metal ion, does not restore the renaturability of the DNA template. However, variation of the duplex 3′-terminal sequence of the template led to significant alterations. In general, GC-rich sites enhanced the displacement of the nascent RNA, while AT-rich sites enhanced formation of the DNA–RNA hybrid. With some terminal sequences, over 85% of the RNA produced by purified calf thymus RNA polymerase II is displaced. Of the DNA homopolymers tested, only dC allows efficient initiation when added to the 3′-OH terminus of a duplex template. This appears to be due in part to the inability of other homopolymer sequences to bind the polymerase efficiently and in part to a requirement for a pyrimidine at the start site for efficient chain initiation. The sites of RNA chain initiation with dC-tailed duplex DNA templates have been mapped by primer extension and S_1 nuclease digestion to the first six nucleotides on the dC tail immediately adjacent to the 3′ end of the template duplex. The sites of initiation are the same for different RNA polymerases and are not affected by whether the transcript is displaced or remains a hybrid.

Protein-coding genes in eukaryotes are transcribed by class II RNA polymerase. While this enzyme plays a vital role in the process of gene expression and has been the subject of much research, it has been difficult to obtain detailed information as to the biochemical processes involved in transcription. This is due to the complexity of the eukaryotic transcription machinery and the difficulty of recreating the transcription reaction in a well-defined in vitro reaction. Purified RNA polymerase II preparations do not use promoters efficiently but depend on nicks and single-stranded regions to initiate transcription. This usually results in random starts and elongation over heterogeneous sequences [reviewed in Lewis & Burgess (1982)]. Specific transcriptional initiation by RNA polymerase II can be achieved in vitro by using cell extracts (Weil et al., 1979; Manley et al., 1980), and fractionation of the components of these extracts that allow RNA polymerase II to recognize and initiate at authentic promoters is in progress (Parker & Topol, 1984; Matsui et al., 1980; Samuels et al., 1982; Davison et al., 1982; Dynan & Tjian, 1983). These systems have already been valuable in deter-

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