

# On the Structural Organization of the Transcriptional Unit in Animal Chromosomes

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Three levels of genome organization in eukaryotes are considered in this paper: (1) the structure of the transcriptional unit (or transcripton); (2) the arrangement of proteins along unfolded chromosomal DNA in chromatin; and (3) the mode of DNA packing in the chromatin.

## The Structure of Transcripton

**The model.** Briefly, according to a model proposed previously (Georgiev, 1969) and slightly modified now (Fig. 1), the transcripton consists of a rather small structural zone containing one or a few structural genes and a large acceptor zone, which does not carry structural information but contains the acceptor sites interacting with structural and regulatory proteins of the chromosome. The structural gene is localized at the end of the transcripton. The whole transcripton, including the acceptor zone, is transcribed into giant precursor of mRNA (pre-mRNA). The initiation and progression of the transcripton is under the control of interaction of regulatory proteins with acceptor sites. Giant pre-mRNA is formed, which consists of the informative part near the 3' end (mRNA) and the noninformative part (pseudo-mRNA). During the processing, the latter is destroyed, whereas the informative part, or true mRNA, is conserved and transferred into the cytoplasm.

Some acceptor sites are reiterated. The identical copies may be present either in the same transcripton or in different transcriptons. The latter allows one regulatory factor to switch on or off many different transcriptons simultaneously.

This model is in agreement with many facts that have appeared during the last years, in particular with some genetic data. Complementation analysis demonstrated that one band in *Drosophila* corresponds to one complementation unit, probably to one structural gene (Judd et al., 1972). On the other hand, the amount of DNA per band for haploid set is much higher than required for coding one protein molecule (Beermann, 1972). In addition,

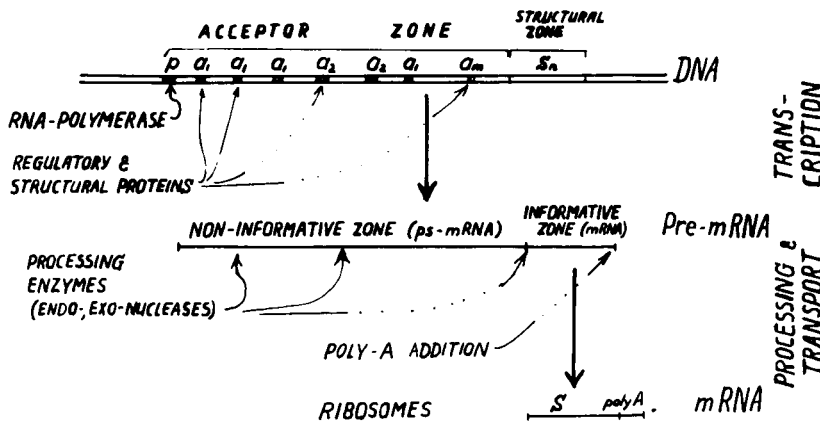
renaturation experiments demonstrated that such structural genes as genes for hemoglobin and fibroin are represented by only one or a few (not more than three) copies per genome (Bishop et al., 1972, Bishop and Rosbash, 1973, Suzuki et al., 1972).

These facts taken together show that a significant portion of DNA in eukaryotes does not carry structural information. However, the main data on the structure of the transcripton have originated from studies on the structure of pre-mRNA.

**Structural organization of pre-mRNA.** Pre-mRNA (synonyms: dRNA, DNA-like RNA; HnRNA, heterogeneous RNA; mlRNA, messenger-like RNA) was discovered in 1962 (Georgiev and Mantieva, 1962) and shown to be a precursor of mRNA. The molecular weight of the nuclear pre-mRNA is much higher than that of cytoplasmic mRNA (Samarina, 1964, Samarina et al., 1965, Yoshikawa et al., 1964; Scherrer and Marcaud, 1965). Competitive hybridization experiments demonstrated that all rapidly hybridizing sequences of mRNA are present in pre-mRNA, although these sequences represent only a small part of all rapidly hybridizing sequences of pre-mRNA (Georgiev, 1966; Arion and Georgiev, 1967; Shearer and McCarthy, 1967; Scherrer and Marcaud, 1968). Lindberg and Darnell (1970) showed that nascent SV40-specific RNA in SV40 transformed cells is a part of giant nuclear RNA, whereas in cytoplasm it is observed as a rather low molecular weight mRNA. Later, poly(A) sequences 150–250 nucleotides long were demonstrated in both nuclear pre-mRNA and mRNA of the cytoplasm (Lee et al., 1971; Edmonds et al., 1971; Darnell et al., 1971), confirming a precursor-product relation between them.

Recently the existence of hemoglobin mRNA sequence in the giant nuclear pre-mRNA from erythroblasts was proved by hybridization of hemoglobin anti-mDNA to giant pre-mRNA (Imaizumi et al., 1973) and also by experiments in which induction of hemoglobin synthesis in frog oocytes occurred upon injection of pre-mRNA (Williamson et al., 1973). In both cases, the data

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**Figure 1.** A slightly modified model of a transcripton in eukaryotes (Georgiev, 1969). (*p*) Promotor; ( $a_1$ - $a_m$ ) acceptor sites,  $m \geq 1$ ; ( $s_n$ ) structural gene(s); usually  $n = 1$ .

indicated that the total amount of mRNA sequences in pre-mRNA is low and corresponds to approximately one mRNA sequence per one giant pre-mRNA molecule.

The next question is: Where is the mRNA sequence localized in the pre-mRNA chain? To answer this question, the hybridization properties of 5'-end and 3'-end sequences of pre-mRNA were studied. 5'-end sequences were detected by the presence of triphosphorylated nucleotide. After alkaline hydrolysis, pppNp group is formed, and these groups were really found in a fraction of giant pre-mRNA ( $S \geq 35$ ) (Ryskov and Georgiev, 1970). 3' ends were labeled chemically using periodate oxydation- $[^3\text{H}]$ borohydride reduction technique (Leppla et al., 1968). After alkaline hydrolysis, labeled nucleoside derivatives were isolated and used as indicators of a 3'-end group (Coutelle et al., 1971).

Double-labeled pre-mRNA was broken to short segments and tested in hybridization with DNA in the presence or absence of nonlabeled mRNA competitor (Table 1). It was found that at DNA-driven  $C_0t$  of about 1-10, both 5'-end and 3'-end

sequences hybridize more efficiently than total RNA, indicating the existence of reiterated sequences at both ends. Polysomal mRNA very poorly competes with giant pre-mRNA, but it strongly inhibits the hybridization of 3'-end sequences. This result indicated the existence of mRNA at the 3' end of the precursor (Coutelle et al., 1970; Georgiev et al., 1972). This main conclusion from experiments on hybridization of end sequences is in good agreement with the above-mentioned model.

As most of the structural genes are nonreiterated, it seems unlikely that the short reiterated sequence at the 3' end is a copy of the structural gene. One could suggest that it is a special auxiliary sequence that functions as a terminator or as a signal for poly(A) addition. This sequence is transferred to the cytoplasm, and its presence in mRNA may explain the hybridizability of some part of mRNA to DNA at low  $C_0t$  values.

The hybridization of reiterated sequences at the 5' end of the pre-mRNA molecule is not inhibited by addition of polysomal RNA (mRNA), i.e., this

**Table 1.** Hybridization and Competition Properties of 5' and 3' Ends of Giant Pre-mRNA from Ehrlich Ascites Carcinoma Cells

		RNA		RNA			
		Nonhybridized	Hybridized	Hybridized in Presence of Polysomal RNA			
		cpm	cpm	% hybridization	cpm or ratio	% hybridization	% competition
5'-end analysis	$^{32}\text{P}$ in pppNp	370	200	35	230	40	0
	$^{32}\text{P}$ in Np	1,380,000	110,000	7.4	95,000	6.4	-14
	pppNp/Np	$0.027 \times 10^{-2}$	$0.18 \times 10^{-2}$	—	$0.24 \times 10^{-2}$	—	—
3'-end analysis	$^3\text{H}$ -end nucleoside	2200	494	18.3	155	5.7	-69
	$^{14}\text{C}$ internal nucleotides	3940	212	5.2	194	4.7	-10
	$^3\text{H}/^{14}\text{C}$	0.56	2.3	—	0.8	—	—

The isolation and labeling of RNA and its hybridization and analysis of 5'-end and 3'-end groups were performed as described earlier (Georgiev et al., 1972). Data of two typical experiments at intermediate  $C_0t$  values.

part of the pre-mRNA does not contain mRNA sequence (Table 1).

3'-end localization of mRNA in the pre-mRNA was confirmed by poly(A) studies. Long chains of poly(A) (~150-250 nucleotides) were found at the 3' ends of mRNA and nascent pre-mRNA. They are added exzymatically to the 3' end of completed pre-mRNA and are conserved upon the transfer of mRNA to polysomes (Edmonds et al., 1971; Darnell et al., 1971; Lee et al., 1971; Mendecki et al., 1972; Molloy et al., 1972b).

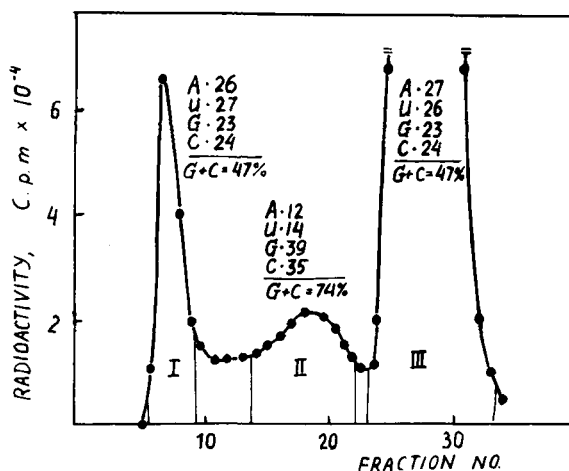
Taken together, these facts prove the 3'-end position of mRNA in pre-mRNA and the absence of mRNA sequences in the 5' part of the precursor. Thus, the structural gene is localized at the end of the transcripton. The beginning of the transcripton does not contain structural genes.

The question arises as to the nature of the non-informative zone. The approach used was to look for specific sequences in the nucleus-restricted part of the pre-mRNA (pseudo-mRNA).

Some of such specific sequences have been discovered during the last years. These are the reiterated base sequences, the main part of which is concentrated in pseudo-mRNA. It is a very heterogeneous population of sequences. Among them, reiterated 5'-end sequences containing pppNp groups should be pointed out (Georgiev et al., 1972), although at this moment they have not yet been purified from other reiterated sequences.

Another type of characteristic sequences is a hairpin-like structure typical of pseudo-mRNA (Ryskov et al., 1972, 1973; Jelinek and Darnell, 1972), some properties of which will be described in the following section. Finally, uridylylate-rich sequences ~30 nucleotides long, also present exclusively in nascent pre-mRNA, were described (Burdon and Shenkin, 1972; Molloy et al., 1972a). All these sequences are degraded in the course of pre-mRNA processing and absent from mature mRNA. Their investigation may give information about the topography and significance of the non-informative part of the pre-mRNA.

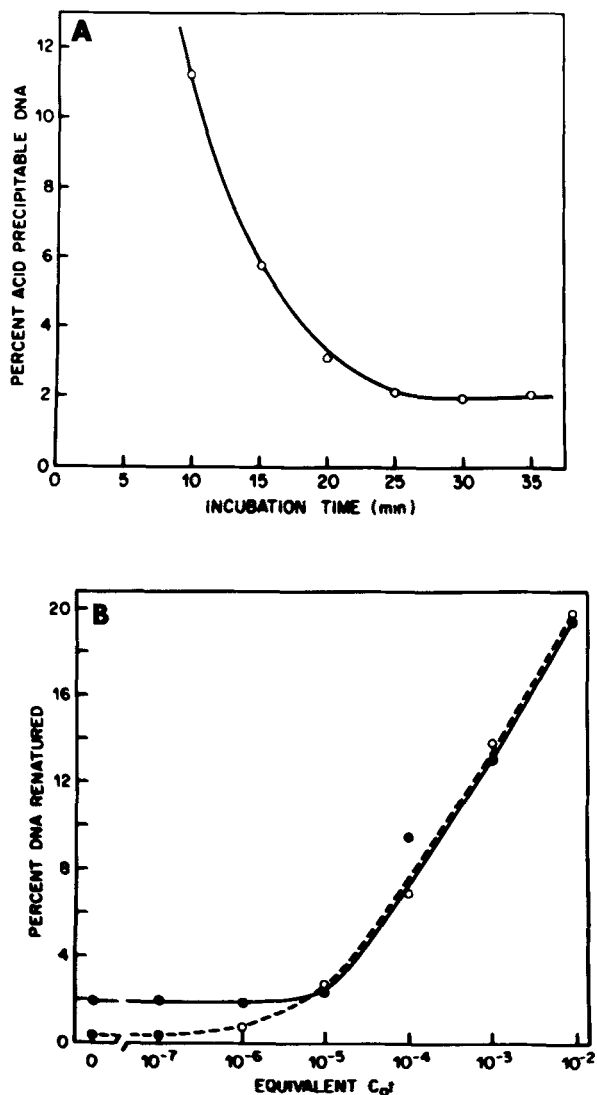
**Double-stranded hairpin-like structures in pre-mRNA.** It was shown previously that pre-mRNA contained sequences resistant to RNase at ionic strength  $\geq 0.1$  (Ryskov et al., 1972, 1973; Jelinek and Darnell, 1972), probably corresponding to double-stranded cellular RNA (dsRNA) discovered by Harel and Montagnier (1971). RNase-resistant material was separated into two peaks by gel filtration on Sephadex G-75 (Fig. 2). Peak I contains poly(A) and long dsRNA segments (~100 base pairs) and peak II, dsRNA of lower chain length (10-20 base pairs). Poly(A) comprises the



**Figure 2.** Isolation of double-stranded, hairpin-like structures from heavy pre-mRNA. Heavy pre-mRNA was isolated from Ehrlich ascites carcinoma cells (labeled with  $^{32}\text{P}$  during 1 hr) with the aid of hot phenol fractionation technique (Georgiev and Mantieva, 1962) followed by sucrose gradient centrifugation. Heavy fraction ( $\geq 45$  S) was collected and treated with a mixture of pancreatic RNase and T1 RNase in  $2 \times \text{SSC}$  during 30 min at  $37^\circ\text{C}$  (Ryskov et al., 1972). The hydrolysate was treated with pronase and then loaded on a Sephadex G-75 column equilibrated with  $0.3 \text{ M}$  acetate, pH 7. The fractions were collected and counted using the Cherenkov effect. The material of peaks I ("long hairpins"), II ("short hairpins"), and III (hydrolyzed material) were collected. Poly(A) was removed from peak I according to Lee et al. (1971). It comprises about 10% of the material. Each sample was hydrolyzed with  $0.5 \text{ N}$  KOH overnight, and base composition was detected on the basis of radioactivity of nucleotides separated electrophoretically.

main part of peak I in the light pre-mRNA (10-30 S), whereas in the heavy pre-mRNA ( $\geq 30-40$  S), the main part of peak I belongs to dsRNA. Poly(A) was separated from dsRNA by the technique of Lee et al. (1971). Both dsRNAs have symmetrical base composition: in peak I it is AU-rich and in peak II very GC-rich.

The double-stranded structure of RNase-stable material was proved by means of chromatography on hydroxyapatite and also in melting experiments. If pre-mRNA untreated with RNase is melted by heating and then rapidly cooled, dsRNA is reconstituted. This suggests the hairpin-like structure of dsRNA. Some other confirmations will be presented below. For this reason, we designate the material of peak I as "long hairpins" and the material of peak II as "short hairpins." Long hairpins account for about 1% of the heavy pre-mRNA. The content of short hairpins is somewhat higher (~2%), and they are present also in the light pre-mRNA. In dsRNA isolated after RNase treatment the loop is nicked, and melting leads to a separation of the complementary strands, thus making possible re-association and hybridization experiments.



**Figure 3.** Demonstration of the reverted repetitions in mouse DNA. Highly labeled [ $^3\text{H}$ ]DNA from mouse L cells was sheared, denatured by heating, then renatured either in 0.12 M phosphate buffer or in 0.18 M NaCl at 60°C for various periods to obtain the  $C_{0t}$  values indicated. After incubation, the sample was diluted with buffer for DNase S1 assay (Sutton, 1971), and DNase S1 was added. After exhaustive digestion, the acid-insoluble material was counted. In some experiments DNA was denatured in the presence of 2% formaldehyde. The latter was then removed in the cold by rapid gel filtration. DNase S1 then was added either immediately or after a short incubation at 60°C. The latter removed methylol groups bound to DNA and allowed reassociation. (A) Kinetics of DNase S1 digestion of denatured DNA after annealing at  $C_{0t} = 10^{-7}$ ; (B) renaturation curve for denatured DNA. (—●—) Denaturation without formaldehyde; (—○—) denaturation with formaldehyde.

Long hairpins are almost completely absent from light nuclear pre-mRNA, suggesting their rapid destruction in the course of processing. Both kinds of hairpins are absent from mature mRNA. Thus they are typical of pseudo-mRNA sequences. The experiments described below were carried out mainly with "long hairpins."

To understand the nature of the hairpins, their hybridization and renaturation properties were studied. It was observed that melted dsRNA from the peaks I and II hybridizes efficiently with DNA at low  $C_{0t}$  values (Jelinek and Darnell, 1972; Ryskov et al., 1973). This indicated its transcription from reiterated DNA base sequences. Approximate calculations for the genome of Ehrlich carcinoma cells gave  $\sim 500$  different families of hairpins, with  $\sim 500$  members in each family.

The next step was the isolation of DNA sequences from which dsRNA was transcribed. It was suggested that they are transcribed from reverted repetitions of DNA. To isolate the latter, extensively sheared, radioactive mouse DNA ( $\sim 500$  base pairs long) was melted and then renatured at very low  $C_{0t}$  values ( $\leq 10^{-7}$ ), excluding the renaturation even of the most reiterated sequences, such as satellites, and allowing only the self-renaturation, i.e., the renaturation of covalently linked complementary strands. Single-stranded DNA was then destroyed by DNase S1, which is absolutely specific for single-stranded DNA (Ando, 1966; Sutton, 1971). Self-renatured sequences survived and were purified from the digested material by hydroxyapatite chromatography. About 2% of the DNA was stable after annealing at extremely low  $C_{0t}$  value (Fig. 3B). According to its behavior in hydroxyapatite chromatography and its melting properties, DNase S1-resistant material is represented by dsDNA. The loop in the DNA hairpin survives the DNase S1 treatment, as the melting and rapid cooling does not make the material digestible and does not change its chromatographic behavior on hydroxyapatite. However, if one melts the DNase S1-resistant DNA in the presence of 1% formaldehyde and then removes free formaldehyde by rapid gel filtration in the cold, it becomes possible to destroy this DNA by DNase S1. Even very short incubation in the absence of formaldehyde (enough to remove methylol groups from DNA) makes the material DNase S1-stable again. The binding of DNA to hydroxyapatite column is changed in the same manner.

It seems very probable that the DNase S1-resistant material isolated from denatured DNA has a hairpin-like structure and encodes hairpins of pre-mRNA. The hairpins in denatured DNA correspond to reverted repetitions in native DNA. According to the DNase S1 test, reverted repetitions account for 2% of the total DNA (Fig. 3A). About half of them may originate from AT-rich satellite, since in purified satellite 10–15% of the sequences survive the treatment with DNase S1 after denaturation. (Another half belongs to the main component.) Their size is of the same order as the size of long hairpins in the pre-mRNA. Short

DNA hairpins were not detected in the experiments mentioned. Thus the size and the content of reverted repetitions in DNA and of long hairpins in pre-mRNA appear to be about the same. One hairpin could be found in the pre-mRNA chain of about 20,000 nucleotides long (mol. wt.  $6-7 \times 10^6$ ), which corresponds to a 1:1 ratio between the number of pre-mRNA molecules and the number of hairpin-like structures. Similarly, one reverted repetition is present per 20,000 base pairs of DNA (mol. wt.  $12-15 \times 10^6$ ).

To elucidate the relationship between DNA and RNA hairpins, hybridization experiments were carried out. DNA hairpins, melted in the presence of formaldehyde, were fixed on nitrocellulose membrane filters and hybridized with denatured dsRNA. In conditions where the RNA-driven  $C_0t$  and DNA-driven  $C_0t$  were of the same order, similar amounts of RNA were hybridized to DNA and renatured (forming RNA-RNA duplexes) (Table 2). This result qualitatively indicates that at least a significant part of DNA hairpins serve as templates for the synthesis of long hairpins present in pre-mRNA.

To understand a possible role of reverted repetitions in DNA and hairpins in pre-mRNA one needs to know more about the sequence complexity of hairpins and their tissue specificity. A convenient approach to answer these questions is the study of renaturation kinetics of denatured dsRNA or DNA.

Experiments have been performed with dsRNA isolated from heavy pre-mRNA of mouse Ehrlich ascites carcinoma, mouse Krebs ascites carcinoma, and mouse liver cells (Fig. 4). One can see that although there are some differences in the kinetics of reassociation between different preparations of

dsRNA, in all cases two main fractions can be resolved: (1) very rapidly reassociating RNA sequences with  $C_0t_{1/2} \sim 2-5 \times 10^{-4}$ ; and (2) a more heterogeneous fraction of RNA renaturing in a broader interval with  $C_0t_{1/2} = 0.1-1$ .

The calculated complexity of the rapidly reassociating dsRNA is about 100 base pairs. Therefore, this fraction probably consists of only one kind of sequences; i.e., it represents a homogeneous material. Of course the kinetic experiments do not give exact figures, but in any case, the number of different hairpins in this fraction can not exceed two or three.

As all tissues studied contain the very rapidly associating hairpins, the question arises whether the hairpins are different or similar in different tissues. Mixtures were prepared containing a small amount of highly  $^3\text{H}$ -labeled hairpins from one tissue and a much higher amount of low  $^{14}\text{C}$ -labeled hairpins from another. The mixtures were then incubated in conditions allowing the reassociation of the rapidly renaturing fraction only in low-labeled hairpins taken in higher concentration. The results obtained (Table 3) show the existence of cross reaction between rapidly reassociating hairpins from different tissues. Thus, at least for rapidly reassociating hairpins, there is low, if any, species specificity.

Concerning the physiological role, if any, of the hairpins, one could consider the following possibilities: (1) reverted repetitions in DNA represent the acceptor sites which interact with regulatory proteins, e.g., rapidly reassociating hairpins may be responsible for switching on or off of genes which are active in most of the tissues of a given organism;

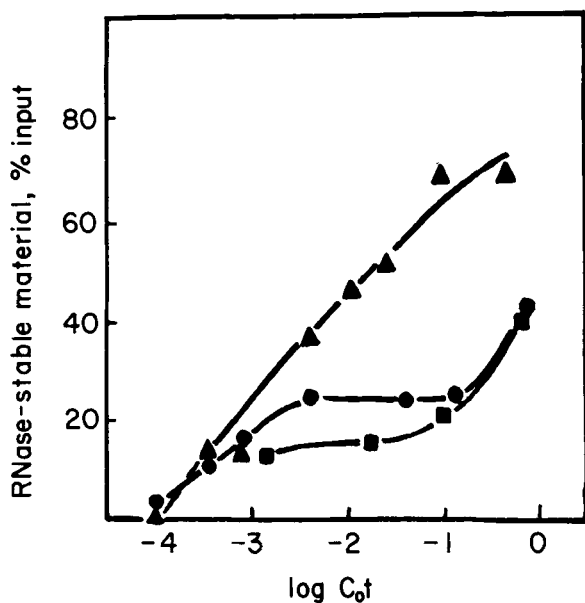
**Table 2.** Hybridization of Long dsRNA Sequences from pre-mRNA Hairpins and from Denatured DNA of Ehrlich Ascites Carcinoma Cells

Exp. No.	Hybridization Conditions				Radioactivity				
	DNA <sup>a</sup> hairpins ( $\mu\text{g}$ )	RNA <sup>a</sup> hairpins ( $\mu\text{g}$ )	Vol. in $6 \times \text{SSC}$ (ml)	Time (hrs)	Hybridized	Nonhybridized		% RNA hybridized to DNA	% RNA Renatured
						Total	RNase-stable		
1	0.70	0.030	0.050	2	390	1000	570	28	41
2	0.30	0.025	0.020	2	350	740	460	32	42
3	no DNA	0.03	0.050	2	5	1500	—	0.3	—
4	total DNA, 70.0	0.13	2.0	0.8	400	6300	—	6	—

RNA-driven  $C_0t$  value was of about 0.05; DNA-driven  $C_0t$  value was of about 0.1.

<sup>a</sup> Specific activities of RNA and DNA preparations were about 50,000 cpm/ $\mu\text{g}$  ( $^{32}\text{P}$ ) and 10,000 cpm/ $\mu\text{g}$  ( $^3\text{H}$ ), respectively.

The double-stranded material from pre-mRNA was isolated as described in Figure 2. The hairpins from denatured [ $^3\text{H}$ ]DNA were isolated as described in Figure 3. Double-stranded DNase S1-resistant material was concentrated and purified by hydroxyapatite chromatography. It was then mixed with nonlabeled, sheared *E. coli* DNA, reprecipitated with ethanol, and denatured by heating in the presence of 3.7% formaldehyde. The solution was diluted with  $6 \times \text{SSC}$  to DNA concentration of 0.5  $\mu\text{g}/\text{ml}$  and passed through an HA-membrane filter several times. The following treatment was according to Gillespie and Spiegelman (1965). RNA was added in  $6 \times \text{SSC}$  and annealed at 65°C. After annealing, the filter was washed several times with  $6 \times \text{SSC}$ , treated with RNase (50  $\mu\text{g}/\text{ml}$ ), again washed with  $6 \times \text{SSC}$  and then with ethanol, and counted. The supernatant was separated into two parts to determine the total and RNase-stable, acid-insoluble radioactivity. All radioactivity measurements for  $^{32}\text{P}$  and  $^3\text{H}$  were made in a SL 40 Counter (Intertechne) using a toluene- $\text{PPO}$ - $\text{POPOP}$  scintillator.



**Figure 4.** Reassociation of denatured long dsRNA sequences (long hairpins) isolated from heavy pre-mRNA of mouse liver and carcinoma cells. Peak I from Sephadex G-75 eluate (see Fig. 2), corresponding to long dsRNA sequences, was extensively purified from RNase by means of SDS-phenol treatment at 65–70°C, precipitated by ethanol with tRNA carrier, collected, dissolved in a small volume of water, put in a sealed capillar, and denatured by heating 15 min in a boiling water bath. The calculation of amount of material was based on the assumption that the specific activity of hairpins is the same as that of the total heavy pre-mRNA. Aliquots of denatured dsRNA were taken, made  $2 \times$  SSC, adjusted by  $2 \times$  SSC to a required concentration, and incubated in sealed capillars for a specified time to the required  $C_0t$ . The concentration of RNA was high enough to avoid incubation longer than 6–10 hr. Samples were then divided, and one-half was immediately put on a glass filter containing 5% TCA, whereas another was treated with RNase mixture (see legend for Fig. 2) and then put on another filter. Both filters were dried by air stream, washed with 5% TCA and ethanol, and counted. Renaturation was determined as a ratio of RNase-resistant activity to the total activity. Background (4–10%) was subtracted in all cases. ( $\blacktriangle$ ) Material from mouse liver; ( $\blacksquare$ ) from mouse Ehrlich ascites carcinoma cells; ( $\bullet$ ) from mouse Krebs ascites carcinoma cells.

and (2) hairpins in pre-mRNA are sites of processing, in particular the sites which separate mRNA from pseudo-mRNA. After cleavage of the hairpin loop these sequences are separated.

To analyze the second possibility, we studied the hybridization of RNA hairpins with the fractions of nuclear pre-mRNA and cytoplasmic poly(A)-containing RNA (mRNA) isolated from Krebs carcinoma cells (Table 4). Denatured  $^3\text{H}$ -labeled dsRNA was annealed at  $C_0t$  values too low for a significant reassociation. To some of the samples [ $^{14}\text{C}$ ]pre-mRNA or mRNA were added, and their influence on the formation of RNase-resistant  $^3\text{H}$ -labeled material was studied. About 20–25% of [ $^3\text{H}$ ]RNA became stable, i.e., formed duplexes with pre-mRNA or mRNA added in excess. The data

presented in Table 4 suggest that both the very rapidly and less rapidly renaturing hairpins are involved in the association with mRNA and pre-mRNA. As pre-mRNA and mRNA are not placed in denaturing conditions before annealing, their own hairpins, if present, should be in the double-stranded form and therefore be unable to reassociate with added denatured dsRNA. One can conclude that single-stranded regions of pre-mRNA and mRNA contain sequences complementary to branches of some hairpins.

The concentration of sequences interacting with hairpins in mRNA is at least not lower than in pre-mRNA. These data are compatible with the suggestion that hairpins separate mRNA and pseudo-mRNA sequences in pre-mRNA, and the processing enzymes cut it just in this position. One of the hairpin branches stays bound to mRNA. Of course, another interpretation is possible, namely, that pre-mRNA and mRNA simply contain sequences complementary to one of the hairpin branches.

In these experiments a new property of pre-mRNA was recognized. A significant proportion of sequences in all fractions of pre-mRNA form RNase-resistant duplexes after annealing (Fig. 5). About 20% of total sequences may be reassociated. This reassociation does not take place at low  $C_0t$ , when satellite sequences reassociate. The main fraction of duplexes is formed at intermediate  $C_0t$  values, but some additional reassociation was also found at very high  $C_0t$  values ( $10^3$ – $10^4$ ). Thus about 20% of the sequences in the pre-mRNA are complementary to each other. This may occur as a result of (1) divergence of reverted repetitions; (2) overlapping of transcriptions which are read in opposite directions; or (3) different directions of transcription in different transcripts containing the same reiterated sequence. In any case, the existence of complementarity between reiterated sequences and possibly between some unique sequences, in pre-mRNA should be considered upon analysis of data of hybridization experiments.

Let us now summarize briefly the main conclusions on the structure of pre-mRNA.

(1) mRNA is localized at the 3' end of giant pre-mRNA.

(2) Besides transcript of the structural gene, mRNA contains some additional service sequences at the 3' end to which poly(A) is added and possibly also some service sequences at the 5' end; the latter may be complementary to one of the branches of some kinds of hairpins.

(3) The noninformative part of pre-mRNA (pseudo-mRNA) contains many specific sequences that do not carry structural information. Among them are reiterated sequences, many of which are complementary to each other.

**Table 3.** Cross Renaturation of Long dsRNA Sequences Isolated from pre-mRNA of Different Mouse Tissues

<sup>3</sup> H]dsRNA Taken in Low Concentration (tissue & C <sub>0t</sub> value)	dsRNA Taken in Excess (tissue & C <sub>0t</sub> value)	Results of Renaturation Reaction			% Renaturation [ <sup>3</sup> H]dsRNA in Absence of dsRNA from Other Tissue		% Similarity of Hairpins
		Total <sup>3</sup> H cpm	RNase-stable <sup>3</sup> H cpm	% Renaturation	low C <sub>0t</sub> <sup>a</sup>	high C <sub>0t</sub> <sup>b</sup>	
Krebs carcinoma	Ehrlich carcinoma	3080	340	11.0	1	25	40
7 × 10 <sup>-5</sup>	4.3 × 10 <sup>-3</sup>	3080	330	10.7			
Mouse liver	Ehrlich carcinoma	1940	300	15.5	0	35	40
8 × 10 <sup>-5</sup>	4.3 × 10 <sup>-3</sup>	1940	240	12.6			
Krebs carcinoma	Mouse liver	2400	480	20.0	3	19	90
9 × 10 <sup>-5</sup>	1.7 × 10 <sup>-3</sup>						

<sup>a</sup> Low C<sub>0t</sub> = 7–9 × 10<sup>-5</sup> and <sup>b</sup> high C<sub>0t</sub> = 1.7–4.3 × 10<sup>-3</sup>, as indicated in the first and second columns.

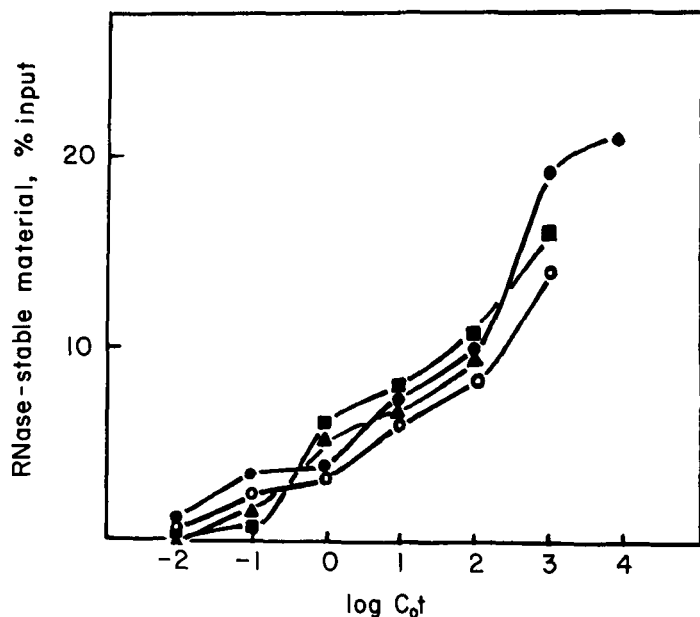
<sup>c</sup> The calculation was as follows: (renaturation in mixture – renaturation at low C<sub>0t</sub>) × 100/renaturation at high C<sub>0t</sub>.

Only very rapidly associating fraction was analyzed (see Fig. 4): Highly labeled <sup>3</sup>H-denatured dsRNA isolated from pre-mRNA of different tissues was mixed with low-labeled, <sup>14</sup>C-denatured dsRNA from other tissues. The renaturation was carried out as described in Figure 4. In two control experiments, highly labeled hairpins were renatured alone. In one of them, C<sub>0t</sub> was the same as for <sup>3</sup>H hairpins in mixture and in another, as for <sup>14</sup>C hairpins in vitro. All figures presented are with background (RNase-stable material at C<sub>0t</sub> = 0) subtracted. It comprises 2–5% in different cases.

**Table 4.** Hybridization of Long Hairpins with pre-mRNA and mRNA of Krebs Carcinoma Cells

C <sub>0t</sub> of hairpins	RNA Added		RNase-stable Material	
	Fraction	RNA added: hairpins	Total (%)	Background (%)
<b>Exp. 1</b>				
1.5 × 10 <sup>-4</sup>	—	—	5	—
1.5 × 10 <sup>-4</sup>	mRNA	2 × 10 <sup>2</sup>	10	5
1.5 × 10 <sup>-4</sup>	mRNA	5 × 10 <sup>2</sup>	14	9
1.5 × 10 <sup>-4</sup>	mRNA	9 × 10 <sup>2</sup>	15	10
1.5 × 10 <sup>-4</sup>	mRNA	94 × 10 <sup>2</sup>	32	27
<b>Exp. 2</b>				
5 × 10 <sup>-3</sup>	—	—	12	—
5 × 10 <sup>-3</sup>	mRNA	24 × 10 <sup>2</sup>	30	18
5 × 10 <sup>-3</sup>	mRNA	96 × 10 <sup>2</sup>	32	20
<b>Exp. 3</b>				
1.5 × 10 <sup>-4</sup>	—	—	5	—
1.5 × 10 <sup>-4</sup>	pre-mRNA, total	9.4 × 10 <sup>3</sup>	29	24
1.5 × 10 <sup>-4</sup>	pre-mRNA, total	94 × 10 <sup>3</sup>	42	37
1.5 × 10 <sup>-4</sup>	pre-mRNA, total (added after annealing, control)	94 × 10 <sup>3</sup>	10	5
<b>Exp. 4</b>				
2.5 × 10 <sup>-2</sup>	—	—	12	—
2.5 × 10 <sup>-2</sup>	pre-mRNA, light	1.2 × 10 <sup>3</sup>	21	9
2.5 × 10 <sup>-2</sup>	pre-mRNA, light	7.3 × 10 <sup>3</sup>	25	13
2.5 × 10 <sup>-2</sup>	pre-mRNA, light	40 × 10 <sup>3</sup>	23	11
2.5 × 10 <sup>-2</sup>	pre-mRNA, light	37 × 10 <sup>3</sup>	28	16
2.5 × 10 <sup>-2</sup>	pre-mRNA, light (added after annealing, control)	37 × 10 <sup>3</sup>	12	0
2.5 × 10 <sup>-2</sup>	pre-mRNA, light (added after annealing, control)	37 × 10 <sup>3</sup>	10	0
2.5 × 10 <sup>-2</sup>	pre-mRNA, heavy	1.25 × 10 <sup>3</sup>	21	9
2.5 × 10 <sup>-2</sup>	pre-mRNA, heavy	12.5 × 10 <sup>3</sup>	28	16
2.5 × 10 <sup>-2</sup>	tRNA (control)	50 × 10 <sup>3</sup>	9	0

Pre-mRNA of Krebs carcinoma cells was isolated by hot phenol fractionation (Georgiev et al., 1972) and separated into fractions with different S values. mRNA was isolated according to Lee et al. (1971) from the same cells. Long dsRNA sequences were prepared from <sup>3</sup>H-labeled, heavy pre-mRNA as described in Figure 2. They were melted by heating and then reannealed (see Fig. 4) alone or with mRNA or pre-mRNA added in excess. mRNA and pre-mRNA were not preheated to avoid melting of double-stranded regions. RNase treatment and counting of RNase-stable material were as described in the legend to Figure 4.



**Figure 5.** Self-annealing of pre-mRNA fractions. Pre-mRNA was isolated from Ehrlich ascites carcinoma cells with the aid of hot phenol fractionation technique and separated by centrifugation in sucrose gradient. The fractions were collected, purified additionally by DNase and hot phenol treatment, dissolved in  $0.01 \times \text{SSC}$ , heated 5 min at  $95^\circ\text{C}$ , made  $2 \times \text{SSC}$ , and annealed for the required time. The total activity was measured in one aliquot; RNase mixture was then added to another aliquot and incubated during 30 min. RNase-resistant material was precipitated by 5% TCA in the presence of carrier RNA. The amount of RNase-resistant material was determined. The amount of RNase stable material just after melting was about 2–5% for different RNA samples, and this background was subtracted from all figures. (—●—) Pre-mRNA of Ehrlich ascites carcinoma cells obtained in temperature interval  $55\text{--}65^\circ\text{C}$  (10–30 S fraction); (—■—) the same, but obtained in temperature interval  $65\text{--}85^\circ\text{C}$  (10–30 S fraction); (—▲—) the same, but heavy fraction, 40–80 S; (—○—) pre-mRNA of mouse liver obtained in interval  $65\text{--}85^\circ\text{C}$  (40–80 S fraction).

(4) Typical for pseudo-mRNA are hairpin-like structures which are transcripts from reverted repetitions in DNA. They may be divided into long and short hairpins. Among long hairpins one could find sequences of different degrees of reiteration. A part of hairpin sequences are complementary to nonpaired reiterated sequences of pre-mRNA and mRNA.

The role of the specific sequences in pre-mRNA is still to be established.

#### Arrangement of Histones along Unfolded Chromosomal DNA

The second level of chromatin structure is the arrangement of protein in linearized (unfolded) deoxyribonucleoprotein (DNP). DNA in chromatin is combined with histones and nonhistone proteins and folded in some unknown way. Linearization of chromosomal DNP may be achieved by treatment with urea (Georgiev et al., 1970) or by extraction with 0.6 M NaCl, which removes histone F1 (Georgiev et al., 1967; Ohlenbusch et al., 1967). However, both techniques induce redistribution of proteins which remain bound to DNA (Ilyin et al., 1971; Varshavsky, Ilyin, and Georgiev, 1973). Recently milder techniques were described which are based on the transfer of some histones from DNP to added free tRNA or DNA. In the presence of EDTA, three histones, namely, F1, F2a2, and F2b, together with most nonhistone proteins, are transferred—in the presence of  $\text{Mg}^{2+}$  ions only F1 and most of the nonhistone proteins are removed from

chromatin (Ilyin et al., 1971; Varshavsky and Georgiev, 1972). The DNP preparation lacking three histones was used in studies on histone arrangement along DNA (Varshavsky and Georgiev, 1972). Long ( $\sim 1 \times 10^3$  base pairs) stretches of free DNA were found, but even in this case, as will be shown below, some redistribution of remaining histones takes place.

For this reason, the experiments described below were carried out with the DNP lacking only one histone (F1)—(DNP<sub>-F1</sub>). The transfer of histone F1 and of nonhistone proteins from the chromatin to the tDNA in the presence of  $\text{Mg}^{2+}$  results in the lowest, if any, redistribution of other histones.

To analyze histone arrangement along DNA, equilibrium centrifugation of formaldehyde-fixed DNP in CsCl density gradient was used (Ilyin and Georgiev, 1969; Ilyin et al., 1970). The buoyant density of DNA is  $1.70 \text{ g/cm}^3$  and of protein,  $1.25\text{--}1.3 \text{ g/cm}^3$ . Therefore, the buoyant density is a function of the protein content of the DNP molecule. Investigation of density patterns of DNP preparations sheared to various lengths of DNA gives information on protein arrangement in the unfolded ("linearized") DNP.

In agreement with previous data (Ilyin et al., 1970; Hancock, 1970), initial untreated chromatin, solubilized by extensive shearing, gives in CsCl density gradient one broad peak with mean density of about  $1.41 \text{ g/cm}^3$  (Fig. 6). No free DNA was found, although of course this applies only to the DNP fragments containing DNA chains of about



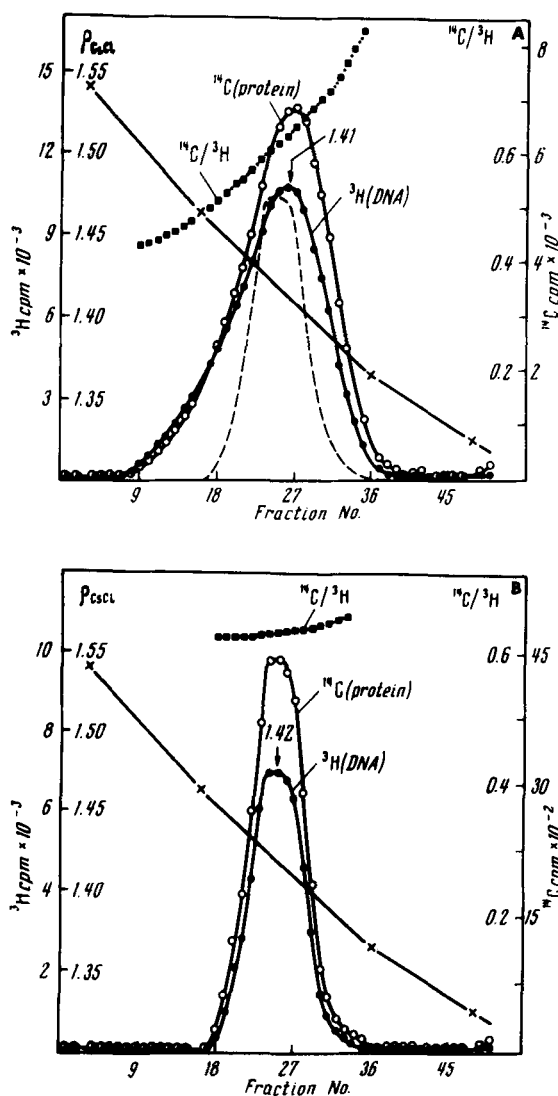
500 base pairs. The existence of shorter blocks of free DNA, as described by Clark and Felsenfeld (1971) and Itzhaki (1971), is not excluded by these experiments. After the removal of histone F1 and of most nonhistone proteins by treatment with tRNA in 1 mM  $MgCl_2$ -1 mM  $Na_2HPO_4$ , pH 7.8, it is also possible to work with almost unshereed DNP samples. Such a DNP<sub>-F1</sub> preparation, containing DNP molecules with average length of  $\sim 10 \times 10^3$  base pairs, gives a sharp peak in CsCl at a density of 1.46 g/cm<sup>3</sup>, and also a significant amount of material is present in the region of higher densities, almost up to the density of free DNA (Fig. 7A). Even after moderate shearing produces rather long chains ( $\sim 4 \times 10^3$  base pairs on average), the second peak already appears with density equal to 1.70 g/cm<sup>3</sup>, i.e., the peak of free DNA (Fig. 7B). At an average chain length of about 500 base pairs (extensive shearing) the

intermediate material between two peaks disappears almost completely and only two sharp peaks are present with densities of 1.70 and 1.46 g/cm<sup>3</sup> (Fig. 7C, D). The amount of DNA recovered as free DNA is about 20% of the total. It should be noted that in the double-labeled DNP preparation (<sup>3</sup>H]DNA; <sup>14</sup>C-protein) no protein is recovered in the peak of free DNA. On the other hand, the protein to DNA ratio in all fractions of the peak "1.46" (Fig. 7A-D) is exactly the same; i.e., this material is almost completely homogeneous.

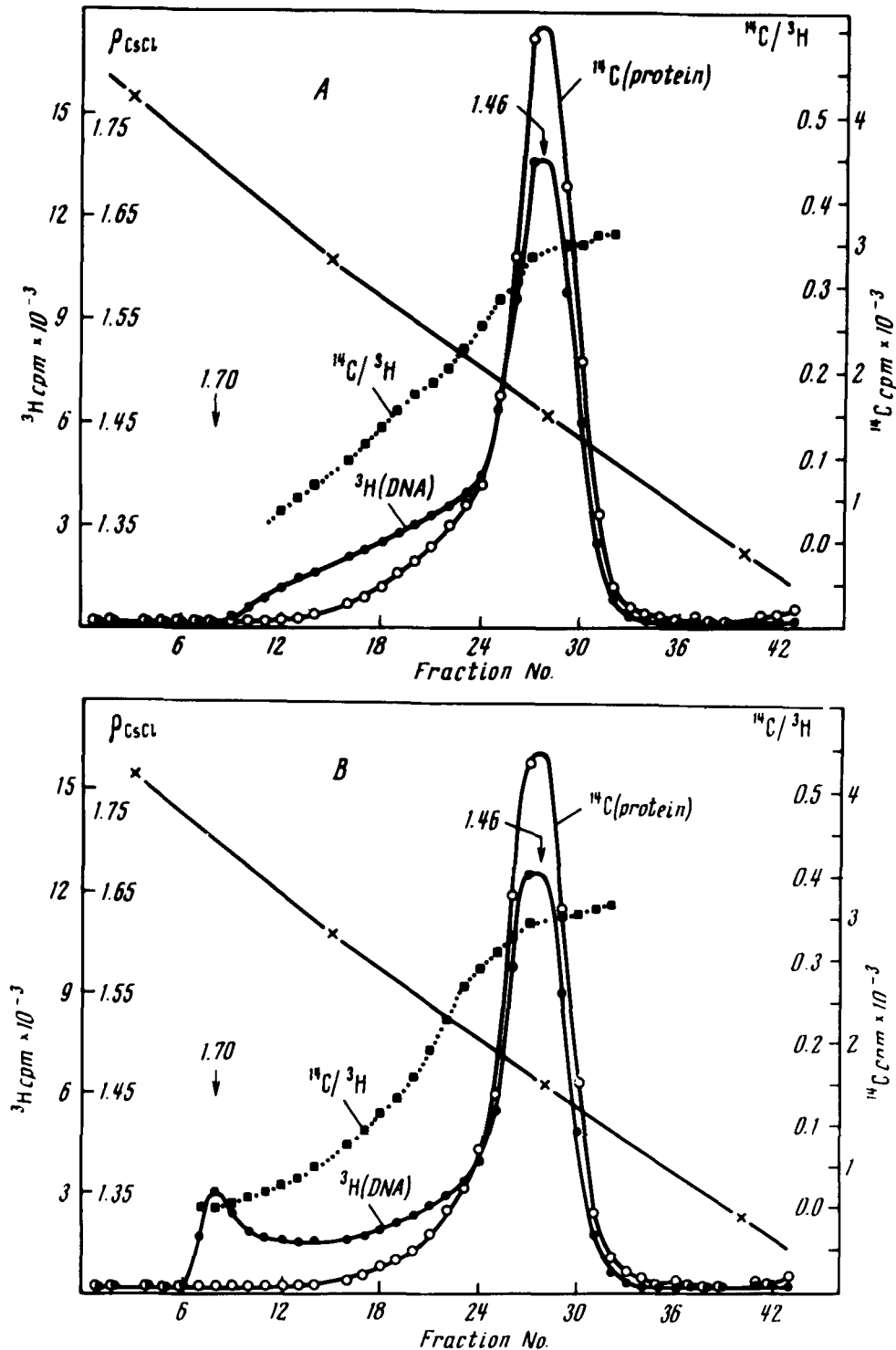
Calculations based on the dependence of density patterns on DNA chain length give average sizes for free DNA and DNP covered with four histones of about  $4 \times 10^3$  and  $16 \times 10^3$  base pairs, respectively.

Some control experiments were carried out to check the possibility of redistribution of proteins during removal of histone F1 and DNP unfolding. In the first experiment the chromatin was extensively sheared before addition of tRNA. Then DNP<sub>-F1</sub> was obtained and analyzed (Fig. 8A). The density pattern was similar to that in the case of DNP<sub>-F1</sub> sheared after tRNA treatment.

Thus the appearance of free DNA after the removal of histone F1 is apparently not due to sliding of the remaining histones along DNA. At the same time, the protein exchange between different DNP molecules in the above conditions was also excluded (Ilyin et al., 1971). For this reason, the observed pattern of protein arrangement along DNA seems not to be a result of histone redistribution.



**Figure 6.** CsCl equilibrium patterns of DNP preparations containing full complement of histones. (A) DNP<sub>0</sub>: Mice carrying Ehrlich ascites carcinoma cells were labeled simultaneously with [*Me*-<sup>3</sup>H]thymidine and L-[<sup>14</sup>C]lysine by six intraperitoneal injections on the fourth and fifth days after inoculation with tumor cells. On the seventh day the cells were collected. Chromatin was isolated as described by Ilyin et al. (1971), washed with 0.3 M NaCl, 0.01 M Tris-HCl, pH 7.8, then with 1 mM triethanolamine-HCl, pH 7.8, suspended in this solution to a concentration of 40–100 μg/ml and then passed through the special press to reduce average length of DNA to  $\sim 1 \times 10^3$  base pairs. The sample was centrifuged at 10,000 g for 30 min (more than 95% of <sup>3</sup>H and 90–95% of <sup>14</sup>C remained in supernatant), fixed with 2% CH<sub>2</sub>O (Varshavsky and Georgiev, 1972), and then centrifuged to equilibrium in the fixed-angle Ti50 rotor at 46,000 rpm for 70–75 hr at 10°C. Fractions were collected directly onto glass filters, which were dried, washed with 7% TCA and ethanol, dried again, and counted with toluene-PPO-POPOP in the computerized SL-40 Counter (Intertechnique). (—X—) Density; (----) equilibrium pattern of DNP<sub>0</sub> treated with 4 M urea before fixation. (B) DNP<sub>0</sub> treated with urea: Chromatin was suspended in 1 mM Tris-HCl, pH 7.8, and then mixed with two volumes of 6 M deionized urea, 1 mM Tris-HCl, pH 7.8. The solubilized DNP was sheared to an average length of about  $1 \times 10^3$  pairs, then dialyzed against several changes of 1 mM triethanolamine-HCl, pH 7.8, fixed with CH<sub>2</sub>O and centrifuged to equilibrium as described above.



**Figure 7.** Arrangement of histones in DNP-F<sub>1</sub>. <sup>3</sup>H,<sup>14</sup>C-chromatin, obtained as described in Figure 6A, was washed and suspended in 1 mM MgCl<sub>2</sub>, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.8. The purified yeast tRNA was added to a concentration of 1 mg/ml and the mixture was incubated with slow shaking for 4–5 hr at 0°C. The swelled gel was solubilized by several strokes in a Dounce homogenizer. DNP-F<sub>1</sub> was separated from free tRNA and from tRNA-protein complexes by gel chromatography on Biogel A-25m. It was fixed in 2% CH<sub>2</sub>O, pH 7.8, for 20–80 hr at 4°C, followed by dialysis against 1 mM Na-EDTA, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.8. Fixed DNP was sheared (by treatment in a Dounce homogenizer, by sonication, or by passing through a press) to a required average length of DNA (the molecular weight of DNA was determined by sucrose gradient centrifugation of DNA in the presence of internal sedimentation markers). The samples of DNP-F<sub>1</sub> were mixed with CsCl and centrifuged at 46,000 rpm for 70–75 hr at 10°C in the SW50 rotor. The gradient was analyzed as described in Figure 6. DNP-F<sub>1</sub> sheared to an average length of (A)  $\sim 10 \times 10^3$  base pairs; (B)  $\sim 4 \times 10^3$  base pairs; (C)  $\sim 1.2 \times 10^3$  base pairs; (D)  $\sim 5 \times 10^2$  base pairs. (—x—) Density.

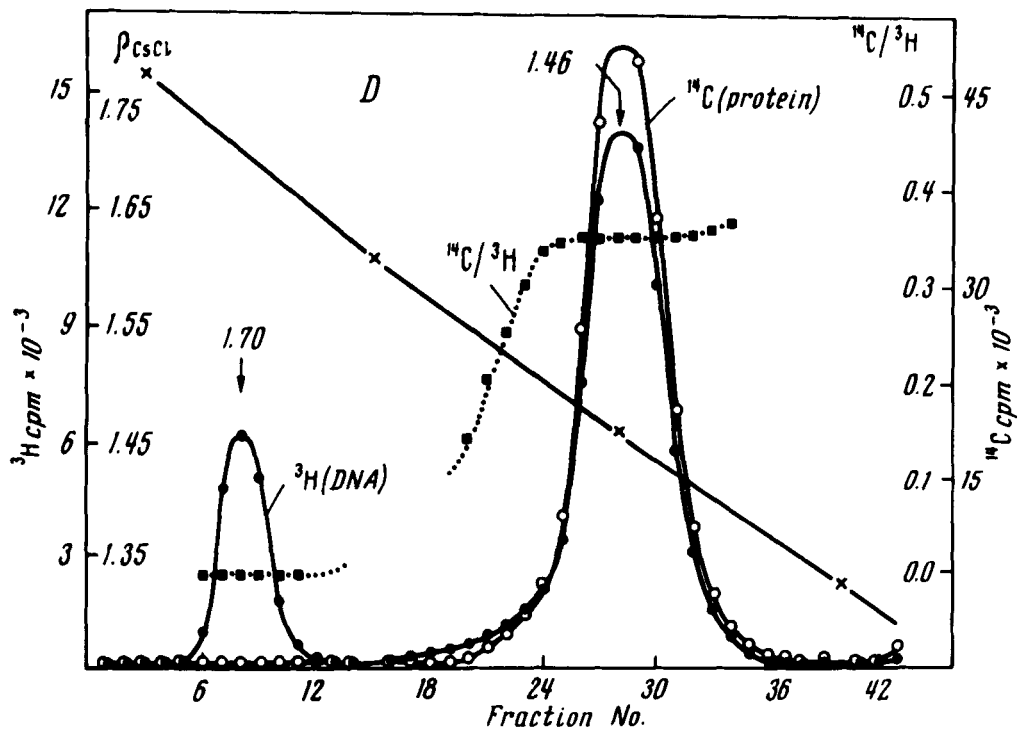
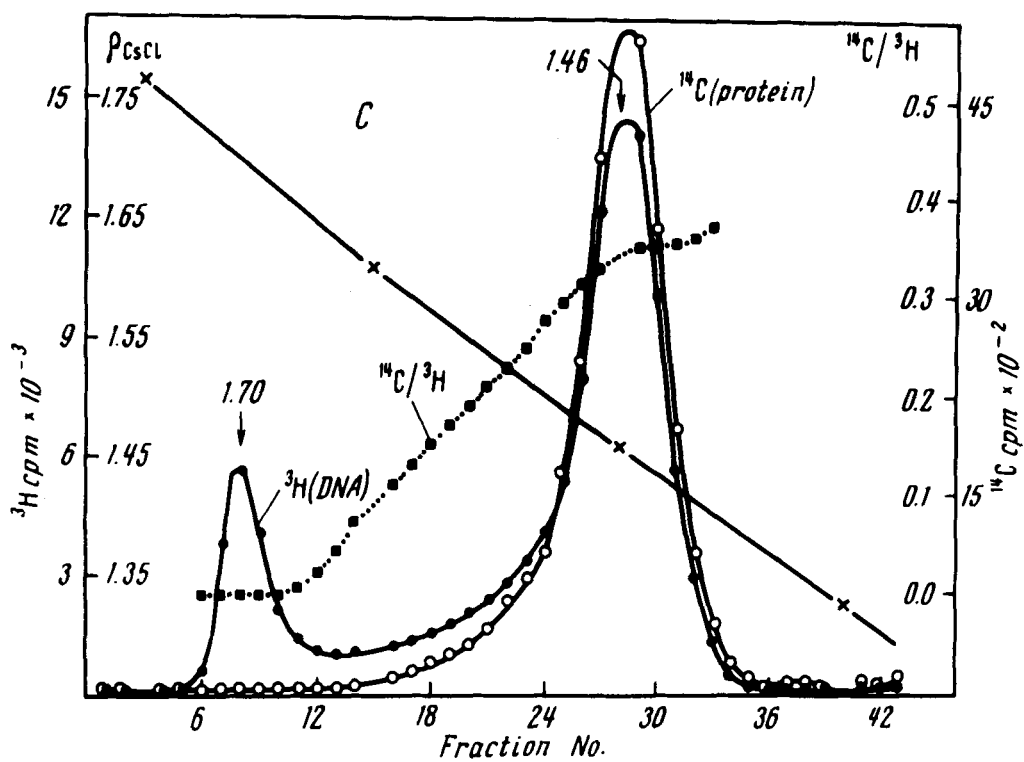
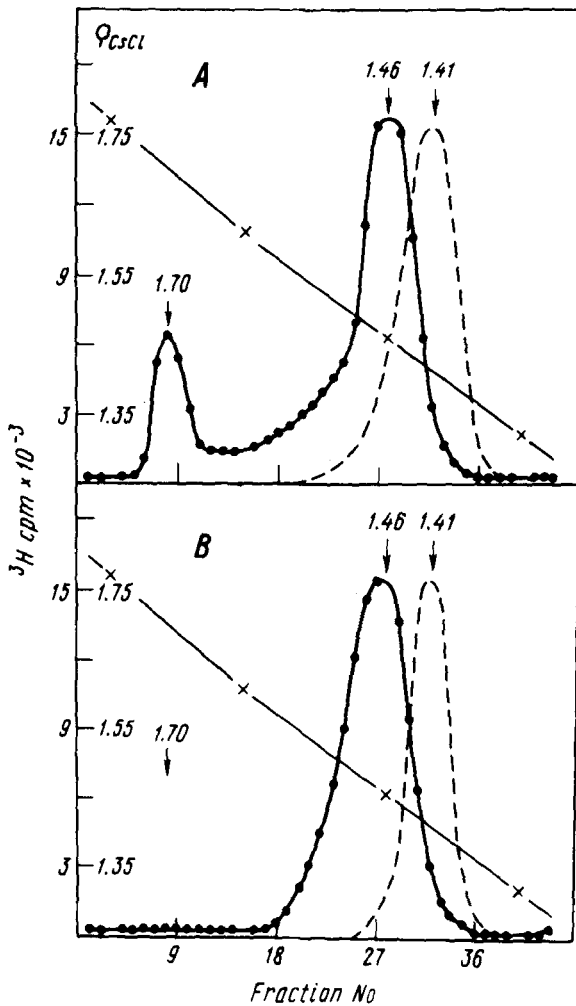


Figure 7 (continued).



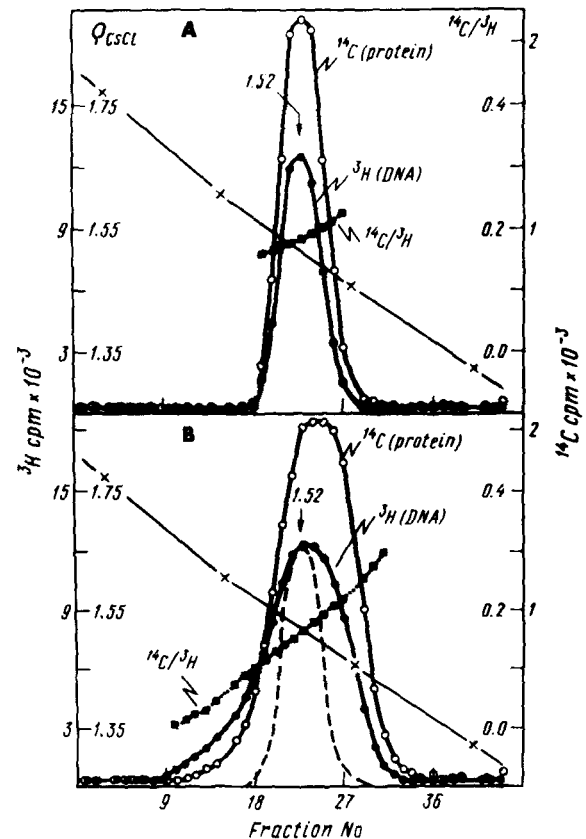
**Figure 8.** The results of some control experiments. (A) Fragmentation of the chromatin before removal of histone F1. Soluble DNP<sub>0</sub> was obtained as described in Figure 6A and then made 1 mM MgCl<sub>2</sub>, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.8. tRNA was then added to 1 mg/ml. The mixture was incubated, then fixed with CH<sub>2</sub>O and analyzed in CsCl density gradient. In separate experiments it was shown that the presence of tRNA and tRNA-protein in the mixture does not interfere with fixation and equilibrium centrifugation distribution of DNP. (B) Influence of protein redistribution on the length of histone-free DNA. Soluble DNP<sub>0</sub> was treated with 4 M urea (see Fig. 6B) with subsequent removal of urea by dialysis against 1 mM MgCl<sub>2</sub>-1 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.8. tRNA was then added to a concentration of 1 mg/ml, and the material was treated as described in (A). (●) [<sup>3</sup>H]DNA; (---) indicates the equilibrium patterns of DNP<sub>0</sub> in (A) and DNP<sub>0</sub> treated with urea in (B).

Another control was to explore the influence of artificially induced protein redistribution on the density pattern of the DNP<sub>-F1</sub>. Urea treatment of the chromatin is known to induce protein redistribution, as can be seen from Figure 6B where the CsCl pattern of DNP treated with urea is clearly different from those of original DNP<sub>0</sub>. Such urea-treated DNP was dialyzed to remove urea and then mixed

with tRNA in 1 mM MgCl<sub>2</sub>-1 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.8. No free DNA was now observed, even after extensive shearing of the fixed DNP samples (Fig. 8B). Thus the redistribution of proteins leads to the randomization of DNP composition and to the disappearance of long protein-free DNA segments.

It is important that the size of the pair—one segment of free DNA plus one segment of histone-covered DNA—is very large (about  $3 \times 10^6 + 12 \times 10^6 = 15 \times 10^6$  daltons), and thus comparable with the size of the average transcript. We suggest that each transcript consists usually of one or sometimes of a few such structural pairs.

If there is any relation between structural units in DNP and functional genetic elements, one could



**Figure 9.** Equilibrium centrifugation of DNA-(F2a1 + F3) in CsCl density gradient. <sup>3</sup>H,<sup>14</sup>C-chromatin was dissolved in 2 M urea, 5 mM Tris-HCl, pH 7.8 (see Fig. 6B) (DNA concentration, 40-100 μg/ml), sheared in a Dounce homogenizer, mixed with extensively sheared unlabeled, double-stranded mouse DNA ( $\sim 5 \times 10^2$  base pairs long) to a final concentration of 1.2 mg/ml and incubated at 0°C for about 5 hr. DNP was separated from complexes of proteins with sheared DNA and from free DNA by sucrose gradient centrifugation, dialyzed, fixed, and centrifuged to equilibrium in a CsCl density gradient (70-75 hr at 10°C in the SW50 rotor). (A) DNA-(F2a1 + F3); average length of DNA is  $\sim 10 \times 10^3$  base pairs. (B) The same, but sheared after fixation to a length of  $\sim 5 \times 10^3$  base pairs. (—x—) Density; (---) indicates the CsCl patterns of (A).

expect that the base sequences, at least the non-repetitious ("unique") base sequences, are different in free and histone-covered DNA. On the other hand, if the formation of free and covered stretches is a purely *in vitro* phenomenon, accompanying DNP unfolding, both fractions should be random and contain all types of base sequences. To check this one should compare the kinetics of reassociation of the total mouse DNA and of the free DNA which was obtained from the DNP<sub>-F1</sub>.

These experiments are in progress now.

### Folding of DNP in Chromatin

The mode of DNA packing in chromatin fibrils remains unclear. The possibilities of superhelical configuration (Pardon and Wilkins, 1972) or double-double helix (Crick, 1971) were considered. We have no direct evidence bearing on the structure of folded DNP, but some data obtained in the course of studies on histone arrangement along DNA and on the pattern of histone redistribution in the chromatin make it possible to suggest some schematic models.

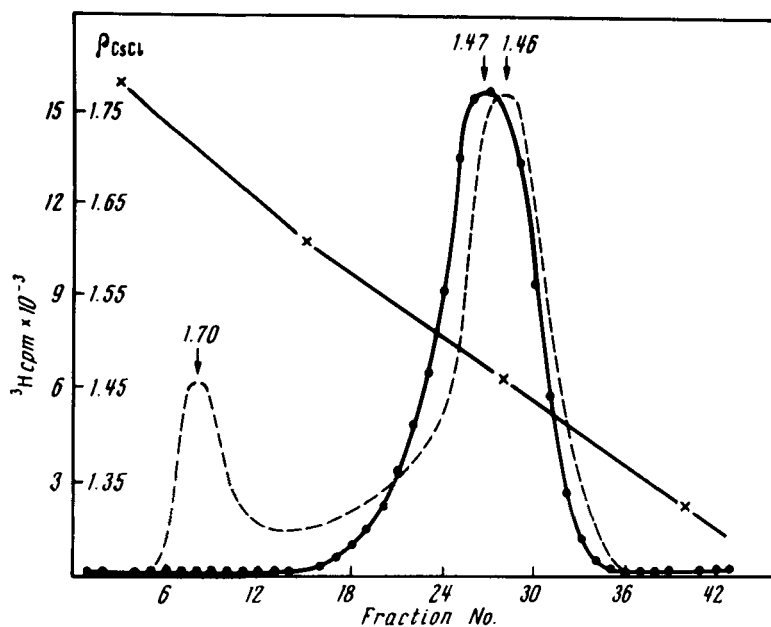
The experimental result is the discovery of dependence of the length of free DNA on the conditions of DNP unfolding which is induced by treatment with tRNA. It is known from previous studies (Ilyin et al., 1971; Varshavsky and Georgiev, 1972) that in preparations of unfolded DNP the transfer of the arginine-rich histones (F2a1 and F3) to added free acceptors (DNA or tRNA) as well as sliding of histones along the DNA strand do not take place (see also above). At the same time, moderately lysine-rich histones F2a2 and F2b are transferred from the DNP to added free DNA (Ilyin et al.,

1971; Varshavsky and Ilyin, unpublished), but this process is very slow and does not interfere with the appearance of free DNA stretches. Thus one could expect that if histones F1, F2a2, and F2b are transferred from chromatin to added free tRNA, the average length of free DNA stretches should be at least the same as after removal of histone F1 only. However this is not the case.

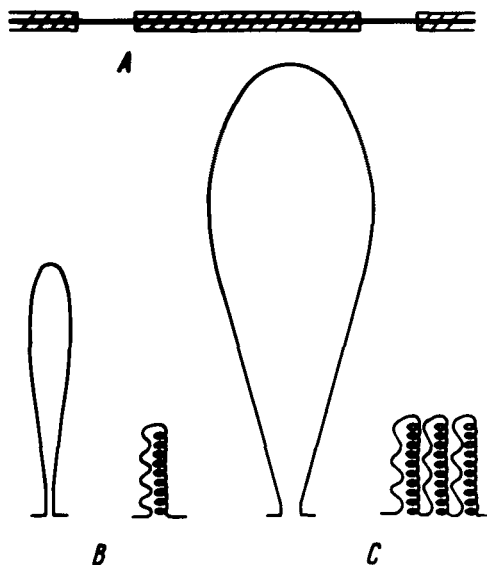
Previously we have found that in DNP containing only histones F2a1 and F3, designated as DNA·(F2a1 + F3), which was obtained by tRNA treatment in the presence of 1 mM Na-EDTA, pH 7.8, the average length of free DNA stretches is equal to 900–1000 base pairs (Varshavsky and Georgiev, 1972). This is at least four times less than in the DNP<sub>-F1</sub>, although the latter DNP preparation is characterized by much higher protein/DNA ratio than the former.

The preparation of DNA·(F2a1 + F3) may be obtained also in another way, namely by treatment of chromatin with excess of sheared double-stranded free DNA in the presence of 2 M urea. In the unfolded DNP, urea does not induce redistribution of the two arginine-rich histones (Ilyin et al., 1971). However, even after extensive shearing of such DNA·(F2a1 + F3) preparations, no free DNA at all could be detected in CsCl density gradient (Fig. 9).

The explanation of these results is that just at the moment of histone transfer from DNP to added tRNA or DNA, accompanied by unfolding of DNA, some part of the histones are shifted from a stretch covered by histones to a free DNA stretch. In the absence of urea, this previously unrecognized type of transfer is completely inhibited by addition of a



**Figure 10.** CsCl pattern of sheared chromatin treated with tRNA in the presence of EDTA. Soluble DNP was obtained by hydrodynamic shearing in 1 mM triethanolamine-HCl, pH 7.8. It was adjusted to 1 mM EDTA, pH 7.8, and tRNA was then added to a final concentration of 1 mg/ml. The subsequent treatment is as described in legend to Figure 9A. (●) [<sup>3</sup>H]DNA; (—x—) density; (---) indicates the equilibrium pattern of the DNP<sub>-F1</sub> obtained from the sheared chromatin in the presence of 1 mM MgCl<sub>2</sub> (Fig. 8A).



**Figure 11.** A model of DNA packing in chromatin. (A) arrangement of four histones (other than histone F1) along unfolded chromosomal DNA; (B) a highly schematic drawing of the mode of DNA packing in a "small" chromomere; (C) a mode of DNA packing in a "large" chromomere. The entire "hairpin" may be additionally folded on itself resulting in a decrease in asymmetry of the whole structure.

sufficient amount of magnesium ions. If  $\text{MgCl}_2$  is added, but its concentration is slightly lower than optimal, the length of free DNA in  $\text{DNP}_{-F1}$  obtained becomes 2–3 times shorter (data not shown).

It should be noted that the same phenomenon is observed if the transfer of histone F1 to tRNA is carried out with the same sheared chromatin sample that gave a lot of free DNA after removal of histone F1 by tRNA in the presence of  $\text{Mg}^{2+}$  (Fig. 8A). No free DNA was found if the removal of F1 was carried out in the presence of 1 mM Na-EDTA (Fig. 10).

We suggest that the transfer of histones other than histone F1 in the course of DNP unfolding depends of the following structural organization of chromatin. Segments of DNA covered with four histones and segments of DNA free from them are brought close together in space, thereby forming two "branches" of a hairpin-like structure (Fig. 11). The branch covered with four histones is packed about four times more tightly than the other branch. It may be supercoiled for example. Histone F1 is responsible for the integrity of the whole structure, combining with either free DNA branch or with both branches of a double "hairpin." One or a few such structural units form one transcription. The activation of the transcription by binding of specific factors (e.g., nonhistone proteins) or by removal or modification of histone F1 leads to the unfolding of a "hairpin." The postulated DNP hairpin should be rather asymmetric, and its average

length should be of about  $1 \mu$ . One can suggest that the entire "hairpin" is additionally folded on itself forming a compact chromomere. During the extensive hydrodynamic shearing of the chromatin gel, the long hairpin may be cut to shorter fragments which behave in the same way as the original long structure upon tRNA treatment with or without  $\text{Mg}^{2+}$  (see above). For final conclusions special structural studies, including X-ray diffraction studies, are necessary.

One of the important questions that remains is the relationship between the three levels of chromosome organization mentioned above, and this question is now being investigated.

#### Acknowledgments

The authors are deeply indebted to M. Eugenev, D. Kramerov, G. Baiszar, V. Farashyan, and S. Limborska for their collaboration in some of the above-mentioned experiments, and to N. N. Dobbert for technical assistance.

#### Note Added in Proof

Recent findings (Varshavsky et al., 1974) confirm and extend the experimental data described above. It was shown that hydrodynamic shearing of chromatin in the presence of  $\text{Mg}^{2+}$  ions (0.6–1.0 mM  $\text{MgCl}_2$ ) produces two discrete types of particles: (a) molecules of completely free DNA which constitute 20–25% of the total nuclear DNA and (b) deoxyribonucleoprotein (DNP) particles which contain all five histone fractions. The average length of free DNA molecules depends on the intensity of shearing and can be as high as 1000 base pairs or more.

Shearing of chromatin in the absence of  $\text{Mg}^{2+}$  ions produces a heterogeneous population of DNP particles; no free DNA is liberated. However, the addition of  $\text{MgCl}_2$  (0.6–1.0 mM) or of NaCl (0.15 M) to such a DNP preparation results in the appearance of free DNA molecules and in a complete restoration of the above "bimodal" distribution.

These findings can be explained in two alternative ways: (1) Each DNP particle in the sheared chromatin (without  $\text{Mg}^{2+}$ ) contains two covalently unlinked DNA molecules and thus corresponds to a fragment of the "asymmetric hairpin" (see Fig. 11). Addition of  $\text{Mg}^{2+}$  results in separation of the two DNA molecules, one of which becomes completely free whereas the other remains histone-covered. (2) Each DNP particle in the sheared chromatin (without  $\text{Mg}^{2+}$ ) contains only one DNA molecule. In the presence of  $\text{Mg}^{2+}$  ions interactions between different DNP particles, for example, during "dimer" formation, lead to an "asymmetric" redistribution of proteins between two DNP particles in the dimer. As a result, dissociation of the

dimer produces one molecule of free DNA and one molecule of DNA associated with increased amount of protein.

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