Kabat, D., and Rich, A. (1969), *Biochemistry 8*, 3742.
Kafatos, F. C. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 1251.
Kirby, K. S. (1965), *Biochem. J. 96*, 266.
Lara, F. J. S., and Hollander, F. M. (1967), *Nat. Cancer Inst. Monogr. 27*, 235.
Lara, F. J. S., Tamaki, H., and Pavan, C. (1965), *Amer. Natur. 99*, 189.
Lowry, O. H., Rosebrough, J. J., Farr, A. L., and Randell, R. J. (1951), *J. Biol. Chem. 193*, 265.
Marmur, J. (1961), *J. Mol. Biol. 3*, 208.
Mejbaum, W. (1939), *Hoppe-Seyler's Z. Physiol. Chem. 258*, 117.
Meneghini, R. (1969), Doctoral Thesis, F. F. C. L., Universidade de São Paulo, São Paulo, Brazil.
Meneghini, R., Armelin, H. A., Balsamo, J., and Lara, F. J. S. (1971), *J. Cell Biol. 49*, 913.
Meneghini, R., Armelin, H. A., and Lara, F. J. S. (1968), *Biochem. Biophys. Res. Commun. 32*, 846.
O'Brien, B. R. A. (1964), *J. Cell Biol. 20*, 521.
Olnes, S. (1970), *Biochim. Biophys. Acta 213*, 149.
Pavan, C., and da Cunha, A. B. (1969), *Annu. Rev. Genet. 3*, 425.
Plagemann, P. G. W. (1969), *Biochim. Biophys. Acta 182*, 46.
Penman, S., Vesco, C., and Penman, M. (1968), *J. Mol. Biol. 34*, 49.
Perry, R. P., and Kelley, D. D. (1966), *J. Mol. Biol. 16*, 255.
Perry, R. P., and Kelley, D. D. (1968), *J. Mol. Biol. 35*, 37.
Rudkin, G. T., and Corlette, S. L. (1957), *Proc. Nat. Acad. Sci. U. S. 43*, 964.
Samarina, O. P., Lukanidin, E. M., Molnar, J., and Georgiev, G. P. (1968), *J. Mol. Biol. 33*, 251.
Simões, L. C. G. (1970), *Rev. Brasil. Biol. 30*, 191.
Solymosy, R., Fedorcsák, I., Gulyás, A., Farkas, G. L., and Ehrenberg, L. (1968), *Eur. J. Biochem. 5*, 520.
Spirin, A. S. (1966), *Curr. Top. Develop. Biol. 1*, 1.

Transcription and Processing of Ribonucleic Acid in *Rhynchosciara* Salivary Glands. II. Hybridization of Nuclear and Cytoplasmic Ribonucleic Acid with Nuclear Deoxyribonucleic Acid. Indication of Deoxyribonucleic Acid Amplification†

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ABSTRACT: Hybridization of nuclear and cytoplasmic RNA with nuclear DNA from *Rhynchosciara* salivary glands, were studied. The results suggested that the salivary gland cells have rapidly hybridizing RNA sequences restricted to the nucleus. Annealing reactions performed with DNA fractionated in CsCl gradients indicated that this exclusively nuclear RNA contains sequences rich in G + C. Apparently the hybridization observed with cytoplasmic RNA is due to reaction of messenger-like species. The results of hybridization reactions with both nuclear and cytoplasmic RNA suggested that there is gene amplification at the time of "DNA puff" development. Hybridization competition did not allow the detection of RNA sequences that are specific to the time of DNA puff development.

In mammalian cells RNA–DNA hybridization indicated that some transcribed RNA sequences are exclusively nuclear (Shearer and McCarthy, 1967; Melli and Bishop, 1969). In these studies for kinetics reasons, the experimental conditions limited the hybridization reaction to repetitive DNA (Britten and Kohne, 1968; Melli and Bishop, 1969), on one side, and to special classes of rapidly hybridizing RNA sequences, on the other (Darnell and Balint, 1970). The function of the repetitive DNA and of exclusively nRNA[1] sequences is very obscure. However, repetitive DNA is of general occurrence in eucaryotes and the same might be true for exclusively nRNA sequences. Consequently the elucidation of their function is very important to understand gene control mechanisms in higher organisms. Unfortunately the hybridization reaction has shown severe limitations to elucidate this question but it still remains as the most simple and powerful technique available to study nucleic acid sequence homology (McCarthy and Church, 1970). It would be desirable to examine in another system some aspects of nRNA that have been studied in mammalian cells. The availability of n- and cRNA from *Rhynchosciara* salivary glands (Armelin and Marques, 1972) opened the possibility of studying hybridization of those RNA fractions with nDNA. Our interest in studying these problems in this tissue comes from: (1) in different stages of larval development salivary glands show different sets of genes activated, at least as far as autoradiography of uridine-*t* incorporation and puff pattern in polytene chromosomes

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[1] Abbreviations used are: nRNA, cRNA, and ml-RNA, nuclear, cytoplasmic, and messenger-like RNAs; SCC, standard saline citrate.

are concerned; (2) the salivary gland DNA presents only 15% of repetitive DNA (Balsamo *et al.*, 1970), which could make the interpretation of results easier; (3) in salivary glands at the end of larval development evidence of gene amplification was found with RNA–DNA hybridization, using a RNA fraction purified and fractionated by a phenol extraction procedure (Meneghini *et al.*, 1971). It would be particularly interesting to approach the following questions. (1) Do the rapidly hybridizing RNA sequences, from nucleus and cytoplasm, change when different genes are activated? (2) Are there RNA sequences restricted to the nucleus? (3) What kind of RNA sequences are transcribed from amplified DNA sequences, whose amplification was suggested by hybridization experiments of Meneghini *et al.* (1971)? In the beginning of this work we faced a difficult problem—the limited knowledge of hybridization reaction available showed that we should not expect straighforward answers to the questions we proposed to ourselves. Moreover our experience with *Rhynchosciara* salivary gland showed that the RNA preparations would be of low specific activity and we would have small amounts of material to work with. However it was possible to obtain results of potential interest for investigators in the field of RNA transcription and processing in eucaryotes.

The hybridization experiments were performed with total DNA in filters (Gillespie and Spiegelman, 1965) or with DNA previously fractionated in CsCl gradient (Birnstiel *et al.*, 1968; Brown and Weber, 1968). We found indications of exclusively nRNA sequences among which some are rich in G + C. Furthermore, the results suggest amplification of some DNA sequences, which are transcribed into nRNA that is partially transfered to the cytoplasm.

Materials and Methods

Animals. A detailed description of the process used for sampling the animals is given in Armelin and Marques (1972). Since DNA from salivary glands of different period larvae were used, we designate as second, third, fourth, and fifth DNA, the DNA of larvae from, respectively, the second, third, fourth, and fifth period as defined in the accompanying paper. The RNA used was from two well-defined larval stages: the beginning of third period, when larvae start making cocoon; and the beginning of fifth period, when the DNA puffs in chromosomes B and C appear. The RNA extracted from these periods will be called third nRNA, and third cRNA for nuclear and cytoplasmic RNA from third-period salivary glands and fifth nRNA and fifth cRNA for nuclear and cytoplasmic RNA from fifth-period salivary glands, respectively.

Uridine-t Incorporation. The incorporation in every experiment was performed "*in vivo*" as described in the accompanying paper (Armelin and Marques, 1972), with uridine-*5-t* (19 Ci/mmole, 1 mCi/ml or 25 Ci/mmoles, 0.5 mCi/ml); injections of 2 μl/larva. Third- and fifth-period larvae show very distinct incorporation patterns as indicated by autoradiography. After 15-min uridine-*5-t* incorporation the density of grains per chromosome area has apparently attained its highest level for both third and fifth period. The main qualitative autoradiographic difference after 15-min incorporation is the high uridine-*5-t* incorporation in DNA puffs of chromosomes B and C and in micronucleoli-like bodies in fifth-period salivary gland; both DNA puffs and micronucleoli-like bodies are not observed in third-period salivary glands (Pavan, 1965; C. Pavan, personal communication; H. A. Armelin and M. C. S. Armelin, unpublished results). For hybridization experiments the RNA was labeled for 210 min with uridine-*5-t*. This incorporation time was considered adequate to label the cytoplasmic ml-RNA and also to obtain homogeneously labeled nRNA. The assumption that nRNA is homogeneously labeled is based on the results of uridine-*5-t* incorporation kinetics (Armelin and Marques, 1972) and also in the observation that after 3-hr incorporation nRNA fractions extracted with phenol at pH 7.4, 0° and at pH 8.3, 65° showed the same specific activity. It is known that the rapidly labeled salivary gland RNA is better extracted at 65° (Armelin *et al.*, 1970; Armelin and Marques, 1972).

Cell Fractionation; DNA and RNA Purification. The methods followed are detailed in Armelin and Marques(1972).

cRNA. cRNA was obtained by direct extraction of cytoplasmic fraction with buffer-saturated phenol–*m*-cresol–8-hydroxyquinoline at pH 7.4 and room temperature, with 1% sodium dodecyl sulfate; the subsequent steps were performed as described in Armelin and Marques (1972).

nRNA. For nRNA extraction, the nuclear fraction pelleted during cell fractionation was suspended in 50 mM Tris-HCl, 50 mM NaCl, 1 mM $MgCl_2$, and 1% sodium dodecyl sulfate pH 8.3 buffer; the suspension at this stage usually shows enormous viscosity. The extraction was performed with buffer-saturated phenol–*m*-cresol–8-hydroxyquinoline at pH 8.3, first at room temperature, and then at 65°. The nucleic acids obtained by both room temperature and hot extraction were routinely mixed after first ethanol precipitation and the following purification steps were performed as for cRNA.

Fractionation of cRNA. In order to obtain 28S rRNA and 9S to 16S ml-RNA, purified cRNA was centrifuged in 10–40% sucrose gradients. cRNA samples were placed over gradients of 25 ml and centrifuged for 16 hr at 23,000 rpm at 4° in a SW25 rotor Spinco ultracentrifuge. After the run the gradients were collected in fractions of 1 ml, aliquots of 0.05 ml of each fraction were taken to determine absorbance profile at 260 nm. The fractions from the left side of 28S rRNA peak were pooled and precipitated with two volumes of ethanol as purified 28S rRNA. The same was done with the fractions between the right side of 18S ultraviolet (uv) peak and the 6S fraction, pooled as 9S–16S ml-RNA.

The spectral relationships for any purified RNA were: 260 nm/230 nm 2.1–2.4 and 260 nm/280 nm 2.0–2.2 in 10 mM Tris-HCl–100 mM NaCl pH 7.4 buffer into which RNA was stocked frozen. The specific activity of the final preparation was obtained by precipitation at 5% trichloroacetic acid, collecting the precipitate by filtration in nitrocellulose filter for radioactivity determination. Before precipitation absorbance was determined at 260 nm assuming that at this wavelength a 45-μg/ml solution of RNA at pH 7.4 has absorbance 1.00. This agrees very well with specific activity measured by alkaline hydrolysis of RNA and subsequent determination of radioactivity in the alkaline hydrolysate.

Hybridization Reaction. We followed the process of Gillespie and Spiegelman (1965) with small modifications.

Denaturation of DNA. The DNA was denatured by addition of an equal volume of 1 M NaOH to 10 mM Tris-HCl pH 7.4 solution at room temperature; the hyperchromicity observed was of 27–30%. After 5 min the solution was neutralized by addition of adequate amount of Tris–NaCl–HCl cold solution to give a final concentration of NaCl equal to 6 × SSC. After that the DNA was immediately placed on nitrocellulose filters in a cold room at 5°.

Immobilization of DNA in nitrocellulose filters. We always used Millipore filters GS of 13-mm diameter. The filters were soaked at least 2 hr in 2 × SSC before use. The DNA was applied to the filters under moderate pressure.

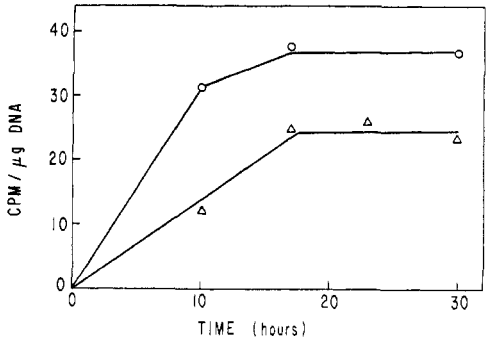

FIGURE 1: Kinetic of the hybridization reaction for cRNA and nRNA. cRNA and nRNA were prepared from salivary glands from third-period larvae. For cRNA the concentration was 250 μg/ml, with specific activity of 410 cpm/μg in 6 × SSC; reaction at 60°; 7 μg of DNA/filter; filters without DNA gave 28–80 cpm/filter. For nRNA: concentration 30 μg/ml, specific activity 2000 cpm/μg, in 6 × SSC; reaction at 65°; 8 μg of DNA/filter; filters without DNA gave 21–28 cpm/filter. cRNA (○) and nRNA (△)

Since filtration took 5–15 min we can assume that renaturation did not take place. After filtration the filters were dried under vacuum for 1 hr at room temperature followed by at least 2 hr at 80°. After immobilization the filters were stored under vacuum at room temperature.

ANNEALING REACTION. The reaction always took place in small volumes. The lowest volume adopted was 0.3 ml but the relative numbers of filters was eight filters per 0.5 ml, in appropriate screw-cap vials. Mineral oil was placed over the RNA solution to prevent evaporation and an extra filter was placed on the top of the set of filters to prevent contact of the reacting filters with the oil. During the reaction the vials were kept in a constant-temperature water bath.

DETERMINATION OF RADIOACTIVITY AND DNA PER FILTER. After reaction the filters were washed four times with 2 × SSC. All filters were put in the same beaker and stirred in a magnetic stirrer for 10 min each washing (ten filters/50 ml). Then the filters were submitted to 10 μg/ml of RNase for 35 min in 2 × SSC at room temperature. After RNase treatment the filters were again washed with 2 × SSC four times, dried at 60°, and counted in a liquid scintillation spectrometer. Following the radioactivity determination the filters were washed four times with chloroform to eliminate the toluene, dried at 60°, and incubated for 15 min in 1 M HCl in boiling water to hydrolyze the DNA. DNA determination was made by absorbance of the acid hydrolysate at 268 nm assuming that a hydrolysate equivalent to 1 mg/ml of DNA has 27.8 of absorbance at this wavelength.

HYBRIDIZATION WITH DNA FRACTIONATED IN CsCl. DNA fractions from CsCl gradient were denaturated by direct addition of equal volume of 1 M NaOH, following neutralization and immobilization in filters as described for total DNA. In general each filter containing one fraction of the gradient was cut in two equal halves to allow hybridization of the same gradient with two different RNA fractions. For annealing reaction all filters corresponding to one gradient were placed in the same vial with the RNA solution. Other steps of the process were made as described for total DNA. In all experiments the DNA per filter was determined for fractions with significant amount of DNA in order to verify the retention and the distribution between the two half-filters of each gradient fraction. In the graphs we plotted the counts per minute of RNA complexed per half-filter because there are several gradient fractions with very small amounts of DNA to be detected with the methods of determination adopted.

TABLE I: Annealing Reaction at Different Ionic Strengths.

Solution	Cpm in Filters with 4 μg of 4th DNA	Cpm in Empty Filters	Cpm Hybrid 6 × SSC: Cpm Hybrid 2 × SSC
	Expt 1[a]		
2 × SSC	335	35	
			1.14
6 × SSC	402	60	
	Expt 2		
2 × SSC	186	12	
			1.26
6 × SSC	236	27	

[a] In expt 1, we used fifth cRNA at 1000 μg/ml with a specific activity of 280 cpm/μg. In expt 2, we used third cRNA at 1500 μg/ml with a specific activity of 100 cpm/μg. In both cases the temperature was 60° and the reaction occurred for 20 hr.

Results

Reaction Conditions. The reaction conditions were selected in order to allow experimentation with labeled RNA of low specific activity. In the conditions finally selected the formation of DNA–RNA complex depended on the type of DNA present on the filter, and showed ribonucleotide sequence discrimination to some extent. The results described below provide information for a better interpretation of the data presented in this paper.

In Table I we have the results of annealing reactions observed at two different ionic strengths. As would be expected, we observed more complex formation in 6 × SSC than in 2 × SSC. The n- and cRNA showed different optimum temperatures for hybridization reaction: the nRNA gave higher values at 65° and the cRNA at 60°. Both n- and cRNA showed reaction kinetics as expected for RNA fractions that hybridize to repetitive DNA; at around 17 hr the reactions is finished (Figure 1). DNA from bacteria and several nucleated cells did not promote retention of RNA when compared to empty filters and filters loaded with *R. angelae* DNA; either in the conditions of cRNA reaction (Table II, exp 1) or in the conditions of nRNA reaction (Table II, exp 2). The complex formed during the reaction was very stable under prolonged incubation of filters in 6 × SSC without RNA, as would be expected. The specificity and efficiency of competition was assayed with RNA from *R. milleri*, a *Rhynchosciara* species closely related to *R. angelae*. The heterologous RNA was unable to compete while the homologous RNA competed to a significant extent at the concentration of 1 mg/ml (Table III). The competition reaction discriminated also between ribosomal sequences and messenger-like sequences, as one can see in the results of Figure 8a.

Annealing reactions of nRNA. Several saturation experiments were done for both third- and fifth-period nRNA, the results obtained in some of them are presented in Figures 2, 3, and 9. With 4–5 μg of DNA/filter, clear saturation plateau

TABLE II: Annealing Reaction of nRNA and cRNA with DNA of Several Organisms.

Filter	DNA	μg of DNA/ Filter	Cpm of Hybrid	Cpm/μg of DNA
		Expt 1[a]		
1	*R. angelae*	6	275	40
2	*E. coli*	4	34	1
3	Chick embryo	10	72	4
4	None		30	
		Expt 2[b]		
1	*R. angelae*	3	120	30
2	*Xenopus laevis*	14	32	
3	HeLa cell	3	25	
4	Chick embryo	10	26	
5	None		30	

[a] We used third ^{3}H-labeled cRNA in the following conditions: concentration 200 μg/ml, specific activity 400 cpm/μg, 17-hr reaction in 6 × SSC at 60°. [b] We used fifth nRNA also after 3-hr incorporation of uridine-*5-t*, in the following conditions: concentration 60 μg/ml, specific activity 500 cpm/μg, 20-hr reaction in 6 × SSC at 65°. In each experiment all filters were placed in the same vial and each value presented in the table is the average between two identical filters. The DNA from *E. coli* was extracted with the method of Marmur (1961); the DNA from chick embryos was prepared by the same process described for *R. angelae* DNA preparations. DNA from *X. laevis* and HeLa cell were gifts of Dr. A. G. Gambarini.

was not obtained until 100 μg of RNA/ml, but the curves showed tendency for saturation. In Figure 2a, we have a curve for the third nRNA and in Figure 3a, a curve for fifth nRNA, which were hybridized to second DNA; reciprocal plots of the same results are presented in Figures 2b and 3b. The points given by reciprocal values can be fitted into two straight lines either for third (Figure 2b) or for fifth nRNA (Figure 3b) which probably reflect two average rates of complex formation. These data agree with the normal behavior of other eucaryotic nuclear RNA, for instance nRNA in HeLa cells (Soeiro and Darnell, 1970).

TABLE III: Specificity of Competition Reaction.[a]

Cold cRNA		
Origin	Concn (mg/ml)	Cpm Annealed as % of Control
R. angelae	1	78
R. milleri	1	100

[a] In this experiment, we used third ^{3}H-labeled cRNA of *R. angelae*, concentration 1 mg/ml, specific activity 400 cpm/μg; the reaction proceeded for 23 hr in 6 × SSC at 60°; 6 μg of DNA/filter. Each result is an average of two experimental values. One-hundred per cent of hybridization is equal to 380 cpm; filters without DNA in the absence of cold competitor gave 90 cpm/filter and in the presence of cold competitor 40 cpm/filter.

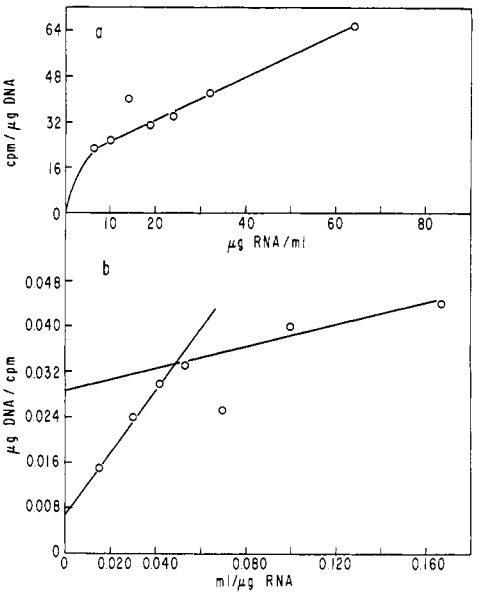

FIGURE 2: Saturation experiment with third nRNA. The annealing reaction took place in the following conditions: 21-hr reaction in 6 × SSC at 65° with 4.0–4.5 μg of second DNA/filter; specific activity of third ^{3}H-labeled nRNA 4200 cpm/μg; filters without DNA gave 21–76 cpm/filter. The values in part b were obtained calculating the reciprocal of the values in part a.

A series of experiments as those described in Figures 2 and 3 was performed and saturation level was estimated by graphic extrapolation with the straight line given by the points of RNA concentration above 20 μg/ml. The data obtained in terms of counts per minute of RNA complexed per microgram of DNA were transformed in percentage of RNA com-

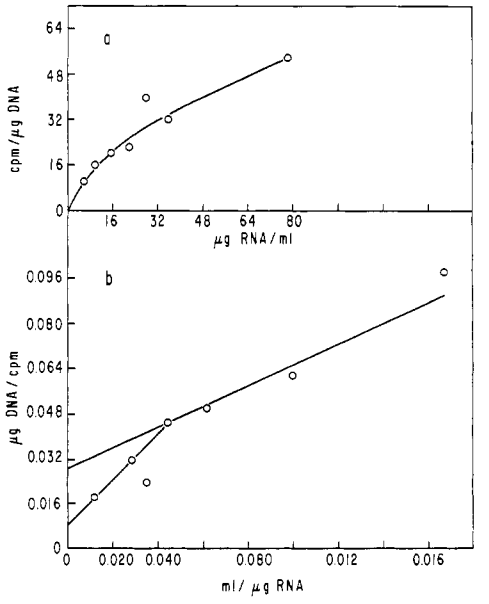

FIGURE 3: Saturation experiment with fifth nRNA. A 20-hr reaction in 6 × SSC at 65°, with 4.0–4.5 μg of DNA/ filter; specific activity of fifth nRNA 2400 cpm/μg; filters without DNA gave 40–90 cpm/filter. The values in part b are reciprocals of the values in part a.

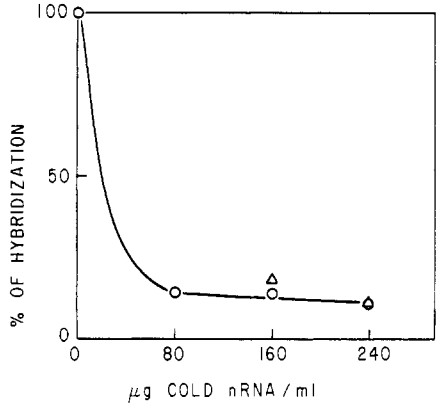

FIGURE 4: Competition between nRNA from the third and fifth period. Fifth ^{3}H-labeled nRNA, 29 μg/ml, with 500 cpm/μg was used in competition experiments with cold fifth RNA (○) and third nRNA (△). A 20-hr reaction in 6 × SSC at 65° with 3 μg of fourth DNA/filter. The 100% of hybridization was determined through an average of two different filters giving 102 cpm/filter, filters without DNA (blanks) showed from 18 to 25 cpm per filter; two filters per point.

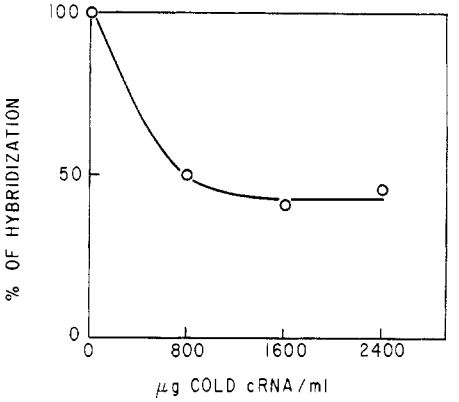

FIGURE 5: Competition between ^{3}H-labeled nRNA and cold cRNA. Fifth ^{3}H-labeled nRNA with 500 cpm/μg, at concentration of 30 μg/ml competed with cold fifth cRNA; 20-hr reaction in 6 × SSC, at 60°, with 3 μg of DNA/filter. The 100% of hybridization was equal to 82 cpm/filter and the filters without DNA showed from 5 to 10 cmp per filter; two filters per point.

plexed with the specific activities of the RNA fractions used (it was assumed that the RNA is homogeneously labeled, as mentioned in Methods) and the estimates obtained are presented in Table IV. The reaction of third nRNA gave a saturation level of 3.3% independently of the DNA used. On the other hand, the reaction of fifth nRNA with second or third DNA gave a value of 5.5%. Certainly, these figures cannot be taken as measures of per cent of genome transcribed. But the different saturation levels suggest that fifth nRNA is more heterogeneous than third nRNA; in other words, fifth nRNA populations should contain sequences not present in third nRNA. However, this interpretation might not be true, because in an ideal case two RNA populations with different sequences and the same complexity can have different hybridization levels, if some sequences in one population hybridize with the largest families of repetitive DNA and sequences from the other population hybridize with the smallest families. If one looks now to hybridization levels obtained with fifth nRNA and fourth DNA, one will notice much higher values. These higher values can be explained by amplification of some DNA sequences from third to fourth developmental period; this interpretation must be true, unless an artefact was involved, but we do not have experimental indication of obvious artificial effects in these experiments. This conclusion will be considered later in connection with other results.

TABLE IV: Percentage of nRNA Annealed to DNA Calculated by Graphic Extrapolation.[a]

	μg of RNA Complexes/100 μg of DNA				
	3rd nRNA			5th nRNA	
DNA	a	b	c	e	f
Second period	3.3		3.1	5.2	
Third period	3.0			5.8	
Fourth period	3.3	4.2	2.9	13[b]	16

[a] The values presented in the table were calculated from results of experiments equally described in Figures 2 and 3. From the reciprocal plot (as in Figures 2b and 3b) we obtained by graphic extrapolation the counts per minute of RNA complexed per microgram of DNA when one over RNA concentration tends to zero. The extrapolated data were determined using the results of hybridizations of RNA in concentrations between 20 and 120 μg per ml with 4–5 μg of DNA/filter, with a minimum of four points per curve. The μg of RNA complexed per 100 μg of DNA was calculated from specific activity of the RNA used. a, b, and c in the table represent three different preparations of third ^{3}H-labeled nRNA with the following specific activities: 4200, 1600, and 2600 cpm per μg, respectively; e and f are fifth ^{3}H-labeled nRNA with 2400 and 500 cpm per μg, respectively. [b] This is not an extrapolated value, it represents an average of two independent single determinations with 100 μg/ml of fifth ^{3}H-labeled nRNA and 5 μg of fourth DNA per filter.

The similarities between sequences contained in third nRNA and fifth nRNA were investigated by competition experiments. The results of a simultaneous competition between cold third nRNA or cold fifth nRNA and fifth ^{3}H-labeled nRNA are shown in Figure 4. These results suggest that there is an identity between third and fifth nRNA in terms of sequence contained. Taking into account the saturation value determined it would be expected that at least cold third nRNA would not be able to compete to the same extent as cold fifth nRNA against fifth ^{3}H-labeled nRNA. This contradiction has two possible explanations: either the stringency of annealing reaction was not high enough to distinguish between different sequences, or some sequences in third nRNA population are in such relatively small concentrations that they were not detected in saturation experiments.

Competition between cold cRNA and ^{3}H-labeled nRNA was also performed. Cold fifth cRNA, in a concentration 80 times higher, reduced the hybridization of fifth ^{3}H-labeled nRNA by 55%, as one can see in Figure 5. In another experiment (not shown), in the same conditions, the competition was lower than in Figure 5 experiment, the hybridization was reduced only by 30%. These results suggested that about 50% of the sequences in nRNA might not be present in cRNA population.

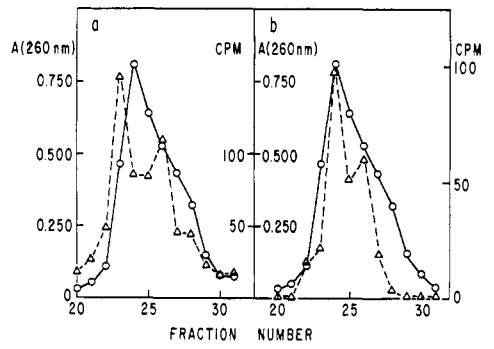

FIGURE 6: Hybridization of third nRNA and third cRNA with DNA fractionated in CsCl gradient. The DNA was obtained from salivary glands and fat body from third-period larva and then it was centrifuged in CsCl gradient given 45 fractions that were placed in nitrocellulose filters as described in Methods. Each filter was cut in two halves to allow reaction with third nRNA (6a) and third cRNA (6b). RNA concentration: third nRNA 30 μg/ml and third cRNA 250 μg/ml, 23-hr reaction at 65° for nRNA and 60° for cRNA. (○) Absorbance at 260 nm; (△) cpm per half-filter.

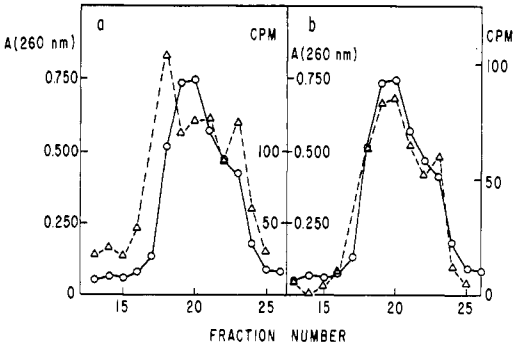

FIGURE 7: Hybridization of fifth nRNA and fifth cRNA with DNA fractionated in CsCl gradient. The experiment was performed exactly as described for third RNA in Figure 6. The CsCl gradient gave only 42 fractions. Fifth nRNA (a) and fifth cRNA (b). (○) Absorbance at 260 nm; (△) cpm per half-filter.

Previous fractionation of DNA in CsCl gradient can improve the resolution of the hybridization reaction (Birnstiel *et al.*, 1968; Brown and Weber, 1968). We performed some experiments annealing nRNA and cRNA with DNA fractionated in CsCl gradient. As one can see in Figures 6b and 7b the radioactivity profile due to counts per minute of RNA complexed follows the absorbance profile for cRNA. But a clear difference appeared for both third and fifth nRNA (Figures 6a and 7a), where significant hybridization occurred at bands with higher densities than DNA main band. These results were observed in seven different experiments, as the one described in Figure 11. It is known that *R. angelae* rDNA bands with higher density than the main band (Meneghini *et al.*, 1971; Gambarini *et al.*, 1970). However the hybridization at higher density bands observed with nRNA cannot be explained exclusively by reaction of nuclear rRNA precursors, because rDNA represents less than 0.1% of total salivary gland DNA (A. G. Gambarini and R. Meneghini, 1970, personal communication). This assumption agrees with a competition experiment between cold rRNA and ^{3}H-labeled nRNA. Competition of cold 28S rRNA at 140 μg/ml, against ^{3}H-labeled nRNA, using nonfractionated DNA in the filter was not detected. This is reasonable, since nRNA should have a considerable amount of ribosomal precursor, but the specific activity is too low. These results indicated to us that the nRNA population might contain sequences rich in G + C restricted to the nucleus and probably distinct from the ribosomal classes.

Annealing Reaction of cRNA. It is known, from observations of several investigators, that the total cRNA or polysomal RNA hybridizes under conditions where only the repetitive DNA reacts (Shearer and McCarthy, 1967; Soeiro and Darnell, 1970; Darnell and Balint, 1971). Some results of annealing reactions of salivary gland cRNA were already described above. These reactions are due to RNA species that sediment between 6 and 16 S. As one can see in Figure 8a, cold 28S rRNA did not show competition capacity against total ^{3}H-labeled cRNA, but cold 6S to 16S cRNA competed very well. In Figure 8b, we present results of competition of cold third and fifth cRNA with ^{3}H-labeled cRNA; the results did not indicate differences between cRNA populations from both larval developmental periods. We performed four independent experiments equivalent to that described in Figure 8b obtaining similar results.

Apparent Amplification for Some DNA Sequences in Fourth and Fifth Period. As shown in Table IV, the extrapolated values for fifth nRNA saturation level varied with "DNA age." The value for fourth- or fifth-period DNA was almost three times the value for second- or third-period DNA. Table IV is useful to describe results of our experiments, however, the figures it includes might contain gross errors if RNA preparations were not homogeneously labeled, as we assumed; nevertheless, our results can be expressed in an independent way. Figure 9a shows hybridization curves of fifth nRNA with DNA from second, fourth, and fifth periods; as one can see the counts per minute hybridized per microgram of DNA increases from second- to fifth-period DNA. Figure 9b shows data from equivalent experiments where third mRNA was hybridized with second and fifth DNA, here we have a very good agreement among values of counts per minute per microgram of DNA which would be expected from extrapolated data of Table IV. The same amplification effect was observed with annealing reaction of ^{3}H-labeled cRNA as one can see in Figure 10, the RNA hybridized gave values of counts per minute per microgram of DNA significantly higher with fifth DNA than with second DNA; the differences however were relatively lower than the differences observed with nRNA. We assumed that the unique explanation for these

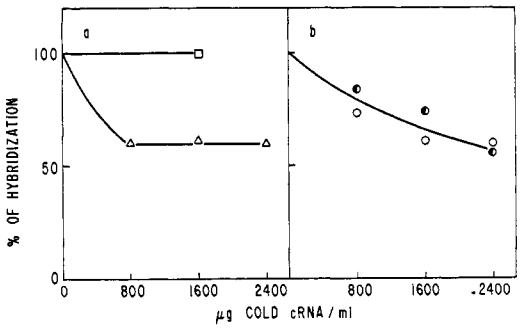

FIGURE 8: Competition between cRNA from third and fifth developmental periods. The radioactive RNA was third ^{3}H-labeled cRNA at 800 μg/ml; 20-hr reaction in 6 × SSC, at 60°; each filter with 3 μg of DNA; 100% of hybridization was equal to 80 cpm/filter; filters without DNA gave 2–12 cpm/filter; two filters per point. Cold competitors: 28S rRNA (□); 6S to 16S cRNA (△); total third cRNA (◐); total fifth cRNA (○).

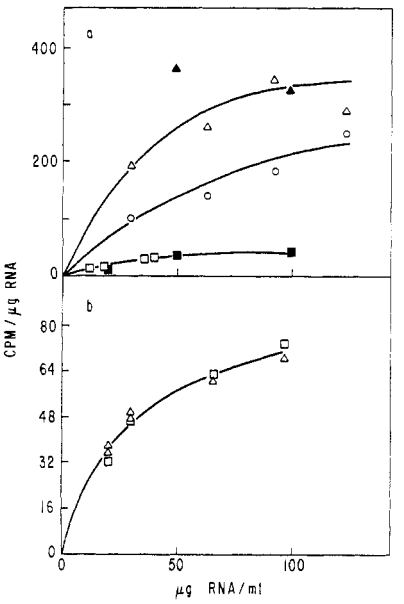

FIGURE 9: Saturation experiments of DNA from second, fourth, and fifth larval period, with fifth and third nRNA. In part a we have the results of hybridization with fifth ^{3}H-labeled nRNA (specific activity 2400 cmp/μg) and in part b the results with third nRNA (specific activity 4000 cpm/μg). Reaction for 20 hr, 6 × SSC at 65°. (□, ■) Two different preparations of second DNA; (○) late fourth DNA; (△, ▲) two different preparations of fifth DNA.

results is an amplification of some DNA sequences (different from rDNA) in salivary glands during the developmental stages between the third and fifth period; the amplified DNA sequences must be transcribed in the nucleus and at least in part be transfered to the cytoplasm.

Interesting results come from hybridization of third ^{3}H-labeled nRNA and fifth ^{3}H-labeled nRNA with fifth DNA fractionated in CsCl gradient. These results are shown in Figure 11, where part a represents third nRNA and part b fifth nRNA; the profile due to third ^{3}H-labeled nRNA hybridized is different of the profile due to fifth nRNA. In order to make the comparison easier the values of counts per minute from part b were multiplied by 3.2 to normalize for specific activities and plotted again in part c with the values of part a. Through the difference between the two radioactivity profiles we obtained the dotted areas in Figure 11c. The same preparations of third ^{3}H-labeled nRNA and fifth ^{3}H-labeled nRNA from experiment of Figure 11 were hybridized with third DNA (not shown). With third DNA the profiles of cpm hybridized were almost identical for third nRNA and

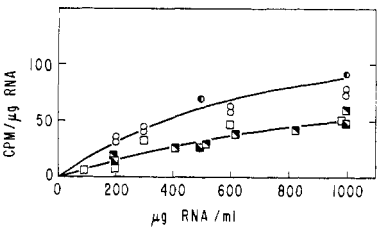

FIGURE 10: Saturation experiment of DNA from second and fifth larval period with fifth cRNA. Fifth ^{3}H-labeled cRNA with 270 cpm/μg; reaction for 20 hr in 6 × SSC at 60°. (□, ◥, ◣) Three different second DNA preparations; (◐, ○) two fifth DNA preparations.

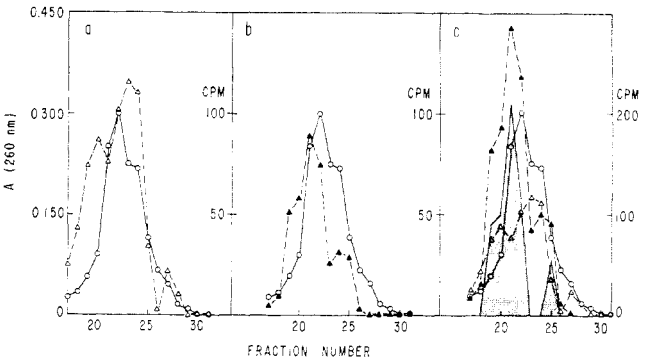

FIGURE 11: Hybridization of third ^{3}H-labeled nRNA and fifth ^{3}H-labeled nRNA with fifth DNA fractionated in CsCl gradient. The CsCl gradient was collected in 45 fractions. The concentration of nRNA was 35 μg/ml; the reaction proceeded for 20 hr in 6 × SSC at 65°. Specific activities: third ^{3}H-labeled nRNA 1600 cpm/μg and fifth nRNA 500 cpm/μg. (○) Absorbance at 260 nm; (△) cpm per half-filter due to third ^{3}H-labeled nRNA, (▲) cpm per half-filter due to fifth ^{3}H-labeled nRNA; in part c: cpm per half-filter due to fifth ^{3}H-labeled nRNA was multiplied by 3.2 to normalize for amount of RNA complexed; (●) represents the difference between the radioactive profiles of fifth and third ^{3}H-labeled nRNA, respectively.

fifth nRNA. We adopt the following interpretation for these results. The dotted areas in Figure 11c might indicate the positions of DNA sequences amplified and transcribed in salivary glands at the fifth period of larval development. Thus DNA amplified sequences band with a buoyant density higher than main DNA band, and therefore must have a higher G + C content and would belong to a relatively restricted class.

Discussion

Significance of nRNA and cRNA Hybridization Reactions. It is known that in the conditions we used for annealing reaction the hybridization is due almost exclusively to reaction of repetitive DNA (Melli and Bishop, 1969). It is obvious that the majority of hybrids formed are not perfect hybrids between sequences of structural genes and mRNA; but we can accept that the hybridization is not simply mismatching due to similarities among sequences of structural genes. It was suggested that repetitive DNA and special classes of RNA might have regulatory functions (Britten and Davidson, 1969). Rapidly hybridizing sequences found in n- and cRNA of HeLa might represent examples of RNA with this vague regulatory function (Darnell and Balint, 1970). In one case a putative regulatory sequence was identified: the recent description of poly(A) sequences in n- and mRNA of mammalian cells (Edmonds *et al.*, 1971; Darnell *et al.*, 1971; Lee *et al.*, 1971). We are assuming that the hybridization in our experiments is due primarily to reaction of RNA sequences of the "regulatory" type.

The hybridization results we described indicate that in salivary gland cells there are RNA sequences restricted to the nucleus; some of these nucleus restricted sequences are rich in G + C and data presented by us in the first paper suggested that the (G + C)-rich sequences are confined to the chromosomes. We must point out that in *Rhynchosciara* a sequence rich in G + C has a relatively lower G + C content than a sequence rich in G + C in mammalian cells. This is because *Rhynchosciara* DNA has 33% G + C and *Rhynchosciara* rRNA has 43% G + C (Meneghini *et al.*, 1971); consequently

a RNA sequence with 40% G + C is a sequence relatively rich in G + C. It is interesting to notice that molecules of heterogeneous nRNA and mRNA in HeLa cells have rapidly hybridizing sequences (Darnell and Balint, 1970); however, rapidly hybridizing sequences from nRNA have higher G + C content than rapidly hybridizing sequences from mRNA (Darnell *et al.*, 1971), and the absolute amounts of poly(A) seem to be the same in heterogeneous nRNA and in mRNA (Edmonds *et al.*, 1971). These results together suggest that processing of mRNA might involve elimination of "regulatory" sequences with relatively high G + C content.

Amplification of Some DNA Sequences. We are assuming that the difference among hybridization results observed for reactions of fifth-period RNA with second, third, fourth, and fifth DNA represent an actual amplification of some DNA sequences. The amplified DNA sequences are transcribed in rapidly hybridizing nRNA and at least part of this RNA is transfered to the cytoplasm. As far as we know *Rhynchosciara* provided the unique evidence so far for amplification of DNA distinct from the very well-known amplification of rDNA (Brown and Dawid, 1968; Evans and Birnstiel, 1968; Gall, 1968, 1969).

Many years ago the DNA puffs were described in *Rhynchosciara* as an example of gene amplification (Breuer and Pavan, 1955; Ficq and Pavan, 1957; Rudkin and Corlette, 1957; Pavan, 1959). Recently it was shown by "*in vitro*" hybridization that the DNA puffs do not contain rDNA (Pardue *et al.*, 1970; Gerbi, 1971). However, the DNA puffs seem to be a special phenomenon restricted to a few species of Diptera (Pavan and da Cunha, 1969). In the case of Diptera *Bradysia hygida*, the development of DNA puffs was inhibited with hydroxyurea but the insect developed normally, showing that the formation of DNA puffs is not a limiting step in larval development (Sauaia *et al.*, 1971). Even if DNA puff in *Rhynchosciara* may be a very specialized event, perhaps rare, its study can provide important information about control mechanisms in eucaryotes. The evidences for gene amplification that we found are restricted to time of DNA puff development; which, however, might be a mere coincidence. First of all, the RNA fractions that we used were preparations of total nRNA whereas DNA puffs represent a small part of the total number of polytene chromosome bands that incorporate uridine-*5-t* in the fifth period of larval development, as indicated by autoradiography.

Also during the fourth and fifth periods there is a burst of DNA synthesis in salivary glands (Simões, 1970; Meneghini, 1969), indicating that the end of polytenization might include complex events. Thus the evidences for gene amplification that we described may not be the consequence of DNA puff development. Other explanations for our results, independently of DNA puffs, would be: during polytenization fractions of repetitive DNA, throughout the genome, become underreplicated which replicate lately in larval development (this is a less probable alternative since fifth RNA hybridizes equally well with second DNA of salivary gland and with ovary DNA, as indicated by results of Meneghini *et al.*, 1971) or at the end of development, fractions of repetitive DNA throughout the genome become overreplicated. Certainly with the present results we cannot decide among these alternative but further studies may provide information of general interest in eucaryotes development.

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References

Armelin, H. A., and Marques, N. (1972), *Biochemistry 11*, 3663.
Armelin, H. A., Meneghini, R., Marques, N., and Lara, F. J. S. (1970), *Biochim. Biophys. Acta 217*, 426.
Balsamo, J., Hierro, J., Birnstiel, M., and Lara, F. J. S. (1970), *Resumos XXII Reunião Anual da S.B.P.C.*, 350.
Birnstiel, M., Speirs, J., Purdom, I., Jones, K., and Loening, U. F. (1968), *Nature (London) 219*, 454.
Breuer, M. E., and Pavan, C. (1955), *Chromosoma 7*, 371.
Britten, R. J., and Davidson, E. H. (1969), *Science 165*, 349.
Britten, R. J., and Kohne, D. E. (1968), *Science 161*, 529.
Brown, D. D., and Dawid, I. B. (1968), *Science 160*, 272.
Brown, D. D., and Weber, C. S. (1968), *J. Mol. Biol. 34*, 661.
Darnell, J. E., and Balint, R. (1970), *J. Cell Physiol. 76*, 349.
Darnell, J. E., Wall, R., and Tushinski, R. J. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1321.
Edmonds, M., Vaughan, M. H., and Nakazato, H. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1336.
Evans, D., and Birnstiel, M. (1968), *Biochim. Biophys. Acta 166*, 274.
Ficq, A., and Pavan, C. (1957), *Nature (London) 180*, 983.
Gall, J. G. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 553.
Gall, J. G. (1969), *Genetics 61, Suppl. 1*, 121.
Gambarini, A. G., Birnstiel, M., and Lara, F. J. S. (1970), *Resumos XXII Reunião Anual S.B.P.C.*, 357.
Gerbi, S. A. (1971), *J. Mol. Biol. 58*, 499.
Gillespie, D., and Spiegelman, S. (1965), *J. Mol. Biol. 12*, 829.
Lee, Se Y., Mendecki, J., and Brawerman, G. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1331.
Marmur, J. (1961), *J. Mol. Biol. 3*, 208.
McCarthy, B. J., and Church, R. B. (1970), *Annu. Rev. Biochem. 39*, 131.
Melli, M., and Bishop, J. O. (1969), *J. Mol. Biol. 40*, 117.
Meneghini, R. (1969), Doctoral Thesis, F. F. C. L., Universidade, de São Paulo, São Paulo, Brazil.
Meneghini, R., Armelin, H. A., Balsamo, J., and Lara, F. J. S. (1971), *J. Cell Biol. 49*, 913.
Pardue, M. L., Gerbi, S. A., Ekhardt, R. A., and Gall, J. G. (1970), *Chromosoma 29*, 268.
Pavan, C. (1959), *Proc. 10th Intern. Congr. Genet. 1*, 321.
Pavan, C. (1965), *Brookhaven Symp. Biol. 18*, 222.
Pavan, C., and da Cunha, A. B. (1969), *Annu. Rev. Genet. 3*, 425.
Rudkin, G. T., and Corlette, S. L. (1957), *Proc. Nat. Acad. Sci. U. S. 43*, 964.
Sauaia, H., Laicine, E. M., and Alves, M. A. R. (1971), *Chromosoma 34*, 129.

Shearer, R. W., and McCarthy, B. J. (1967), *Biochemistry 6*, 283.

Simões, L. C. G. (1970), *Rev. Brasil. Biol. 30*, 191.

Soeiro, R., and Darnell, J. E. (1970), *J. Cell Biol. 44*, 467.

Electrophoretic Study of the Polypeptides from Surface Membranes of Mammalian Cells†

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ABSTRACT: Surface membranes isolated from mouse fibroblasts (L cells) and baby hamster kidney cells before (BHK_{21}/C_{13}) and after (C_{13}/B_4) transformation by an RNA virus were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The optimal conditions for solubilization and electrophoresis of the isolated surface membranes were established. The molecular weight distribution of the polypeptides was determined by using proteins of known molecular weight comparably treated. All of the surface membranes examined show several polypeptides in the molecular weight regions of 230,000–200,000 and a spectrum from 190,000 to approximately 15,000. Polypeptides from the surface membranes of L cells in the molecular weight regions of 230,000, 210,000, 100,000, and 56,000–39,500 contain carbohydrate on the basis of staining with periodic acid–Schiff reagents. These polypeptides stained most intensely with Coomassie Blue. In contrast, the higher molecular weight polypeptides from the surface membranes of BHK_{21}/C_{13} and C_{13}/B_4 fibroblasts, although staining intensely with Coomassie Blue, contain no detectable carbohydrate by the periodic acid–Schiff procedure. The polypeptides of 100,000 and 56,000–39,500 stain with these reagents in a manner similar to those of the surface membranes from the L cells. This lack of detectable carbohydrate in some of the polypeptide regions is discussed in relation to the trypsinization procedure which is used to remove the baby hamster kidney cells from the monolayer cultures. The polyacrylamide gel electrophorograms of the surface membranes of mouse fibroblasts and baby hamster kidney cells are similar. However, after virus transformation, the polypeptides of 210,000, 96,000, 82,000, and 56,000–46,000 are diminished when compared to those of the control membranes.

Proteins and glycoproteins of the surface membrane represent enzymatic, structural, and receptor molecules responsible for many of the functional properties of animal cells. Receptors for viruses, hormones and drugs, cellular recognition sites, and histocompatability antigens are among the specialized functional properties of animal cells which can be assigned to specific surface membrane components (see Kraemer, 1971, for review).

Some of these surface membrane molecules may be associated with the regulation of cell growth. Changes in the glycoprotein and glycolipid composition of the cell surface have been reported for cells after transformation by a number of oncogenic viruses (Wu *et al.*, 1969; Meezan *et al.*, 1969; Buck *et al.*, 1970, 1971; Hakomori and Murakami, 1968; Mora *et al.*, 1969).

Current concepts suggest that proteins and glycoproteins of the surface membrane may function in a mobile state in a fluid lipid bilayer (Singer and Nicolson, 1972). This dynamic nature of the surface membrane has been supported by recent findings demonstrating the mobility of the membrane components (Blasie and Worthington, 1969; Frye and Edidin, 1970; Marchesi *et al.*, 1971; Scott *et al.*, 1971; Taylor *et al.*, 1971).

The characterization of the polypeptides from surface membranes has proceeded slowly, primarily due to the technical difficulties associated with surface membrane isolation with inherent problems of defining the final product (Warren and Glick, 1971), and the subsequent solubilization and fractionation of the membrane components (Fairbanks *et al.*, 1971; Neville and Glossmann, 1971; Trayer *et al.*, 1971). Methods have been developed permitting the isolation of whole surface membranes from animal cells grown in tissue culture (Warren and Glick, 1969). The isolated membranes have been characterized morphologically (Warren *et al.*, 1966) and chemically: lipids (Weinstein *et al.*, 1969), glycolipids (Weinstein *et al.*, 1970), and carbohydrates (Glick *et al.*, 1970). In this study the polypeptides from these mammalian cell membranes were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Materials and Methods

Cell Culture. Mouse fibroblasts (L cells), baby hamster kidney cells (BHK_{21}/C_{13}), and BHK_{21}/C_{13} transformed by the Bryan strain of the Rous sarcoma virus (C_{13}/B_4) were grown and harvested as described previously (Warren and Glick, 1969; Buck *et al.*, 1970). The cultures were examined for the presence of *Mycoplasma* at routine intervals and were found to be negative.

Preparation of Surface Membranes. Surface membranes were prepared by the zinc ion procedure (Warren and Glick, 1969). The isolated whole membranes were counted in a hemocytometer and protein was determined by the method of Lowry *et al.* (1951).

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