Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 2947–2951, May 1982
Developmental Biology

Gene amplification in *Rhynchosciara* salivary gland chromosomes

(cDNA clones/DNA puffs/C chromosome)

DAVID M. GLOVER*, ARNALDO ZAHA, ANN JACOB STOCKER, ROBERTO V. SANTELLI, MANUEL T. PUEYO, SONIA MARIA DE TOLEDO†, AND FRANCISCO J. S. LARA‡

Instituto de Quimica, Universidade de São Paulo, Caixa Postal 20780, São Paulo, Brasil

Communicated by Joseph G. Gall, February 1, 1982

ABSTRACT **Late in the fourth larval instar, several regions of the *Rhynchosciara americana* salivary gland chromosomes undergo "DNA puffing." We have constructed a library of cloned cDNAs synthesized from poly(A)$^+$RNA isolated from salivary glands during the period of development when the DNA puffs are active. From this library we have studied clones representative of three genes active during this period but not active at earlier developmental periods of the gland. One of these genes is not amplified during the developmental process and encodes a 0.6-kilobase RNA molecule. The other two genes are located within the DNA-puff sites C3 and C8 and encode 1.25-kilobase and 1.95-kilobase RNA molecules, respectively. We estimate from the quantitation of transfer hybridization experiments that each of these genes undergoes 16-fold amplification during DNA puffing.**

Gene amplification in somatic cells was first detected by morphological criteria in the larval salivary glands of flies of the family Sciaridae. Several regions of the *Rhynchosciara americana* polytene chromosomes were found to show a type of puffing in which, after puff regression, there was more DNA in the bands involved compared with neighboring bands as indicated by Feulgen staining (1). This was later confirmed both by spectrophotometric measurements (2) and by autoradiographical studies on the incorporation of [^{3}H]thymidine (3). Subsequently, similar observations were made on the salivary chromosomes of larvae from the genus *Sciara* (4–6).

The DNA puffs, which appear in late fourth instar in *Rhynchosciara* salivary glands, are involved in the production of messenger RNAs (7, 8). These encode several peptides of the communal cocoon, which are needed in large amounts over a short period of time (9, 10). Based on morphological and physiological criteria, the fourth instar of *R. americana* larvae has been divided into six periods (11). The first indication of DNA puff formation is the appearance of a fast-green staining band between two orcein (+) bands at the chromosomal sites of these puffs early in period 4 (ref. 12; unpublished data). Amplification and puff formation are subsequently maximal at different times for each site and are also dependent on the position of the cell within the gland (13, 14). The largest puffs are found in region 2 of the B chromosome and in regions 3 and 8 of the C chromosome. The B2 puff is formed preferentially in the first 50 cells of the gland in period 5, whereas C3 attains its largest size in the middle and distal section of the gland in period 6. The C8 puff is similar in all regions and is also maximal in period 6. As the larvae progress from period 3 to period 4, there is a dramatic change in the pattern of RNA and protein synthesis. This consists of an inhibition of rRNA synthesis (15) and the synthesis of new poly(A)$^+$RNA species (7, 8). This is accompanied by inhibition of the synthesis of certain peptides and the synthesis of new ones (14, 16). It is possible at this time to isolate poly(A)$^+$RNAs from the salivary glands that hybridize *in situ* to the B2 (8) or the C3 puff (unpublished data). These RNAs direct the synthesis, *in vitro*, of polypeptides corresponding to those synthesized *in vivo* when these puffs are maximally active (14).

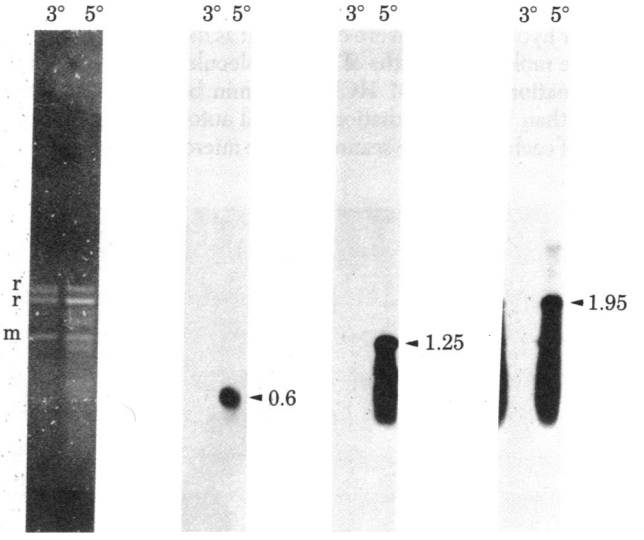

FIG. 1. Three developmentally regulated transcripts represented in the cDNA library. Poly(A)-enriched RNA, corresponding to ≈250 μg of unfractionated RNA from the salivary glands of periods 3 and 5 larvae, was fractionated by electrophoresis on a 0.8% agarose gel containing 10 mM methyl mercury hydroxide. Ribosomal RNA that was not removed by passage over oligo(dT)-cellulose can be seen on the ethidium-stained gel and is labeled r. RNA from the large ribosomal subunit from mitochondria (m) is also enriched by this procedure. The RNA was transferred onto diazobenzyloxymethyl-paper and successively hybridized with a number of ^{32}P-labeled cDNA clones. The autoradiograms show that the principal hybridization given by ^{32}P-labeled pRa3.46, pRa3.65, and pRa3.81 is to RNA molecules from period 5 of 0.6, 1.25, and 1.95 kb, respectively.

Quantitation of the amplification of DNA puffs from *Rhynchosciara* has been attempted by nucleic acid hybridization experiments (ref. 17; unpublished data). The problems with these experiments arise from the use of impure hybridization probes. In this paper we describe the cloning of DNAs complementary to transcripts from two DNA puffs on the C chromosome of *R. americana* and the use of these clones to assess the degree of gene amplification.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: kb, kilobase(s).

* Permanent address: Cancer Research Campaign, Eukaryotic Molecular Genetics Research Group, Dept. of Biochemistry, Imperial College, London SW7 2AZ, England.
† Permanent address: Instituto de Quimica, Universidade Estadual de Campinas, Campinas, S.P., Brasil.
‡ To whom reprint requests should be addressed.

MATERIALS AND METHODS

Construction of cDNA Clones. Double-stranded cDNA was synthesized from poly(A)$^+$RNA from the salivary glands of period 5 larvae (8) by the procedures of Smith *et al.* (18). The cDNA was fractionated by chromatography on Bio-Gel A-50, and dG residues were attached to the ends of the cDNA from the high molecular weight fractions. This dG-tailed cDNA was hybridized with *Eco*RI-cleaved pAT153 (19), which had been tailed with dC residues and introduced into *Escherichia coli* HB101.

Preparation of DNA. DNA was extracted from salivary glands as described by Balsamo *et al.* (20). Plasmid DNA was prepared as described (21).

Transfer Hybridizations. RNA transfer hybridizations were carried out as described by Smith *et al.* (21). Quantitative DNA transfer hybridizations were carried out as described (22) except that the molecular lengths of DNA molecules were reduced by depurination in 0.25 M HCl for 30 min before denaturation rather than by UV irradiation. Several autoradiographic exposures of each filter were scanned in the microdensitometer, and standard curves were constructed from these exposures, giving a linear autoradiographic response in proportion to increasing amounts of plasmid DNA. However, the autoradiograms shown in Fig. 4 are overexposed to facilitate photographic reproduction. Probes were labeled with ^{32}P to specific activities greater than 10^8 cpm/μg by nick-translation.

RESULTS

Cloning DNAs Complementary to Developmentally Regulated mRNAs. Transcripts originating from the DNA puffs are sufficiently abundant that they can be isolated as distinct peaks of RNA sedimenting on sucrose gradients. We reasoned, therefore, that such transcripts would be highly abundant in a library of cloned DNAs constructed from poly(A)$^+$RNA prepared from glands of fourth-instar larvae in period 5, when most of the DNA puffs are active. We made such a library and screened it for colonies that would hybridize strongly with [^{32}P]DNA complementary to RNA from period 5 glands but that would not hybridize with [^{32}P]DNA complementary to RNA from period 3

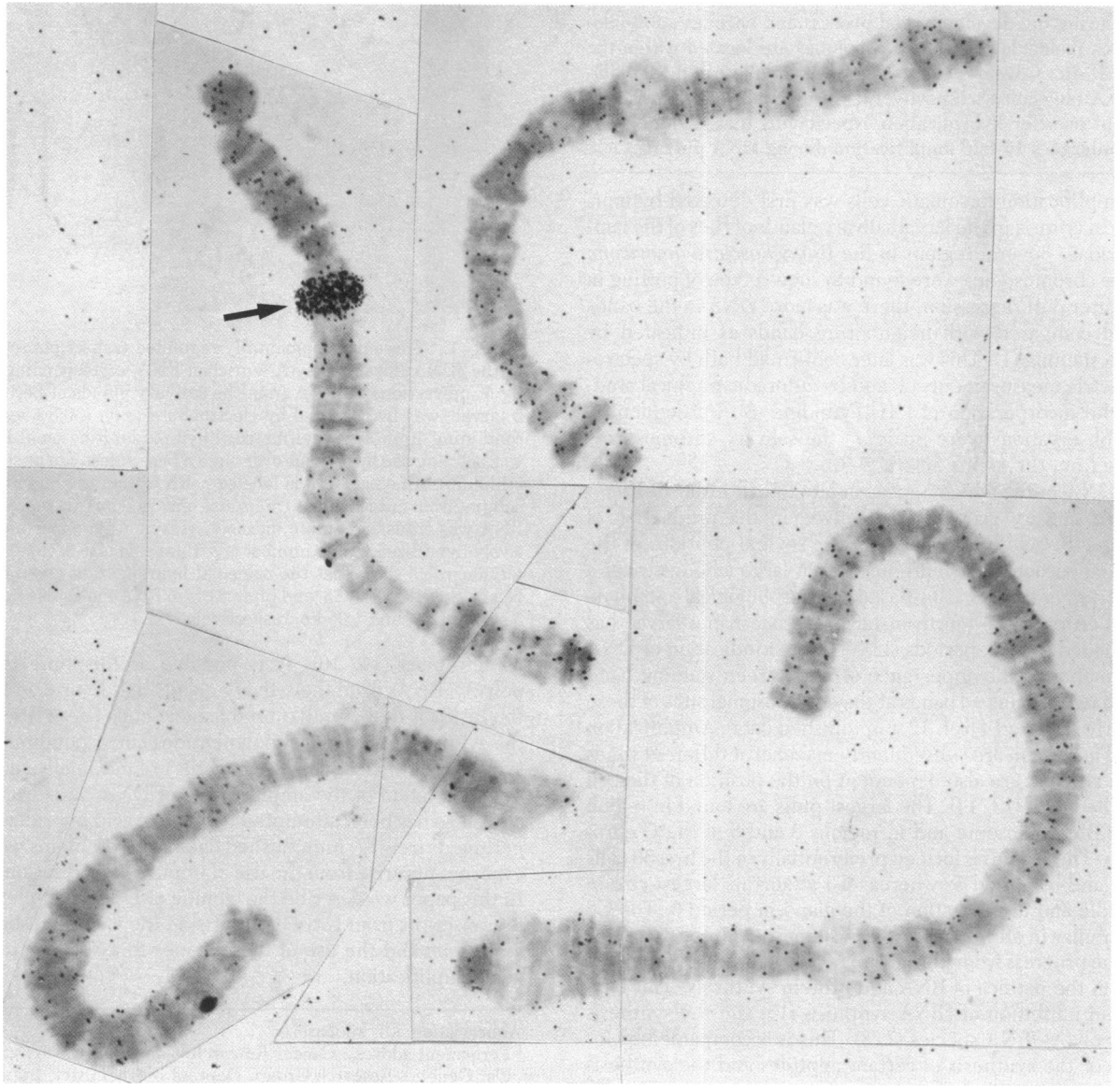

FIG. 2. *In situ* hybridization of pRa3.81. [^3H]cRNA was synthesized from the plasmid pRa3.81 and was hybridized with a squashed preparation of salivary gland chromosomes from period 6 of fourth-instar larvae by the procedures of Stuart and Porter (23). The time of exposure is 2 wk. The arrow points to the C8 puff, the principal site of hybridization.

glands. In this way we were able to eliminate clones of non-developmentally regulated genes and, by picking colonies giving an intense autoradiographic signal, to select plasmids complementary to abundant mRNAs. We screened 600 colonies from the library in this way and selected about 80 that had these properties.

The developmental changes in the activity of three cloned genes selected by the screen are seen in Fig. 1. In this experiment, RNA preparations from salivary glands of periods 3 and 5 were enriched for poly(A)$^+$RNA and then fractionated on denaturing gels. The appearance of several new bands of RNA can be faintly discerned on the stained gels. When the RNA was transferred onto diazobenzyloxymethyl-paper for hybridization with representative clones, we could readily detect three species of RNA of 1.95, 1.25, and 0.6 kilobase(s) (kb) present in RNA from period 5 but not from period 3.

***In Situ* Hybridization of Cloned cDNAs to DNA Puffs from the C Chromosome.** The cDNAs inserted in the majority of the recombinant plasmids fall into two categories on the basis of their restriction endonuclease cleavage patterns. Of 24 clones that we examined in this way, 13 are exemplified by pRa3.65, which is complementary to the 1.25-kb RNA, and 3 by pRa3.81, which is complementary to the 1.95-kb RNA. We localized these sequences on the *R. americana* salivary gland chromosomes by the *in situ* hybridization technique. Fig. 2 shows a set of chromosomes that have been hybridized with the plasmid pRa3.81, in which the site of hybridization is to the DNA puff at C8. Fig. 3 shows the hybridization given by the plasmid pRa3.65; in this case, the main site of hybridization is to C3, the site of one of the major DNA puffs. Other sites are also labeled by this plasmid but to a lesser extent (see legend to Fig. 3). These all correspond to regions that are known to bind poly(U) (24) and that also show quinacrine-bright fluorescence (unpublished data). Other plasmids, pRa3.59 and pRa6.20, that also hybridize to C3 did not show this pattern of hybridization to other regions. We presume that some of the poly(A) tail of the mRNA was incorporated into the cloned cDNA of pRa3.65 and hybridized to these dA·dT-rich regions.

Quantitation of the Gene Amplification in the C3 and C8 Puffs. We determined the copy numbers of several cloned cDNAs from the C3 and C8 puffs in *Eco*RI-cleaved salivary gland DNA from period 3 (designated Ra3°) before puffing occurs and from period 6, when the C3 DNA puff reaches its maximum size (designated Ra6°). The autoradiographic response of the genes in the salivary gland chromosomes was quantitated relative to known amounts of cDNA plasmids in gel-transfer hybridization experiments (Fig. 4). The gene that encodes the pRa3.46 sequences (Fig. 4*A*) is contained within a 16-kb *Eco*RI fragment that appears not to undergo amplification during the transition from period 3 to period 6. It is an example of a gene that can achieve developmental activity remaining as a single copy relative to the majority of the chromosomal DNA sequences. A very different result was obtained with plasmid pRa3.65. Fig. 4*B* shows the hybridization given by this plasmid, which contains a 0.61-kb cDNA insert. We estimate that the cDNA sequence of pRa3.65 was amplified from one copy in period 3 to 15 copies in period 6. We carried out a similar experiment with the plasmids pRa1.66 and pRa6.85, which contain the same cDNA transcripts but of varying lengths, and from these data we calculated levels of amplification of 14-fold and 16-fold, respectively. Fig. 4*C* shows the hybridization of pRa3.81, which has a 0.9-kb cDNA insert. This plasmid is one of two plasmids complementary to the 1.95-kb RNA from the C8 puff for which we carried out such experiments. It hybridized to a 3-kb *Eco*RI fragment encoding a sequence that amplified from one copy in period 3 to 14 copies in period 6. A second clone of these sequences, pRa1.31, contained a longer cDNA insert and hybridized not only to this 3-kb *Eco*RI fragment but also to a 6.2-kb fragment. In this experiment we calculated a 16-fold amplification of the coding sequences in each of these fragments (data not shown).

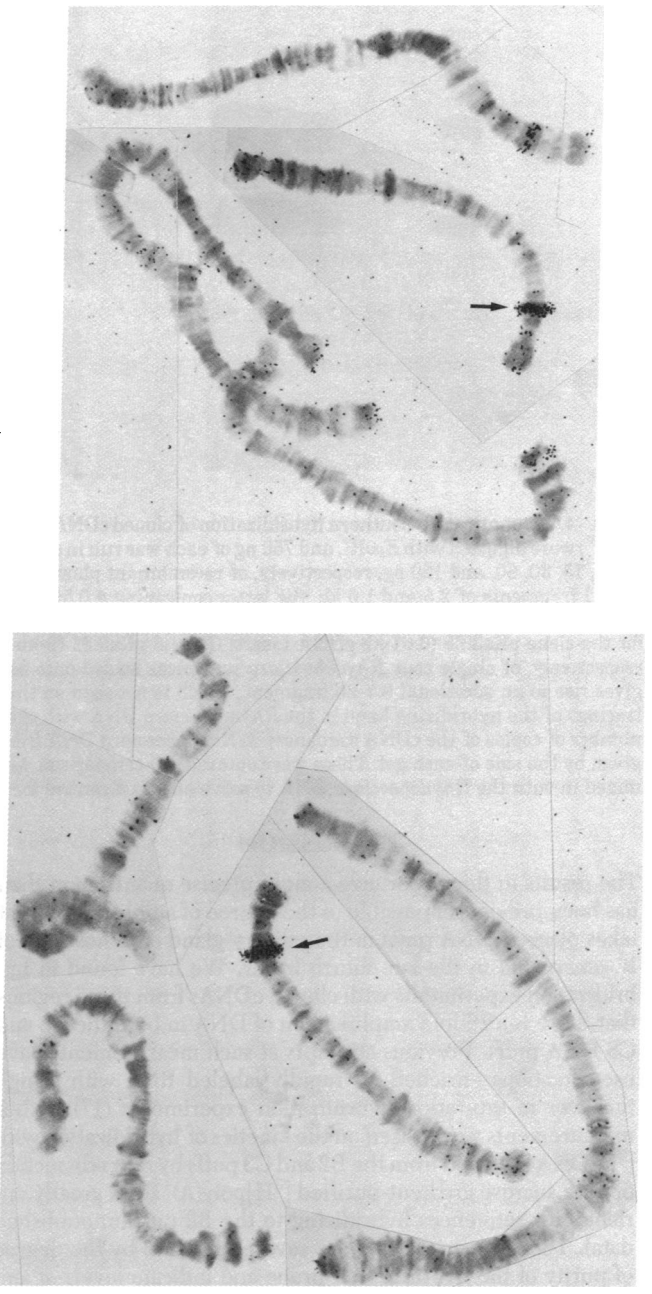

FIG. 3. *In situ* hybridization of pRa3.65. [^{3}H]RNA synthesized from pRa3.65 was hybridized with period 6 salivary gland chromosomes, and the slides were exposed for 1 wk. The main site of hybridization at the C3 puff (C3d) is indicated by the arrow. There are several other sites of hybridization on the chromosome in *A*. (*A*) These are known to be dA·dT-rich regions (see text), principally at C1a (telomere), C2e, C11 (centromeric heterochromatin), B1a (telomere), B2a, B13a-c, B15 (centromeric heterochromatin), X1a (telomere), X2h-j, X3h (not obvious here), X12 (centromeric heterochromatin), A1a-b (telomere), A11–12 (centromeric region), and B18 (telomere). The plasmids pRa3.59 and pRa6.20 also hybridize *in situ* to the C3 puff but do not show hybridization to these dA·dT-rich regions, whereas plasmid pRa3.56 shows the same pattern of hybridization as pRa3.65 does. (*B*) Another set of chromosomes hybridized with cRNA from pRa3.65, in which C3 is preferentially labeled.

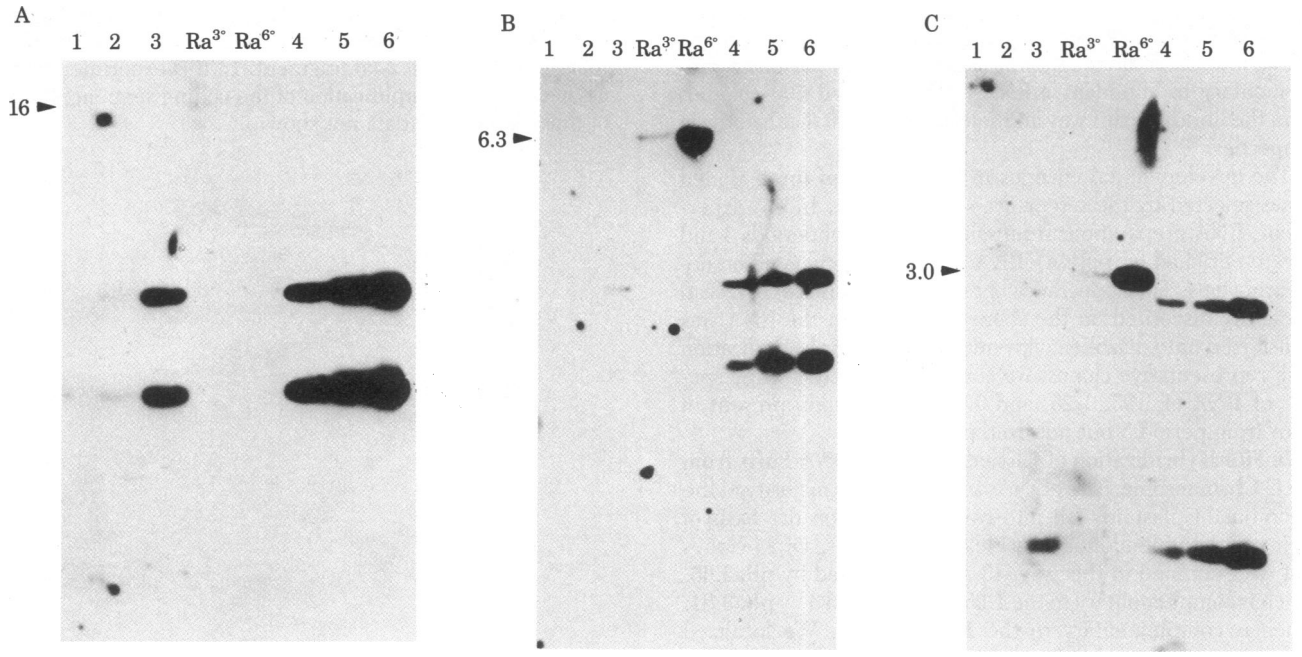

FIG. 4. Quantitative Southern hybridization of cloned cDNAs to salivary gland DNA. Salivary gland DNAs from period 3 ($Ra^{3°}$) and from period 6 ($Ra^{6°}$) were digested with *Eco*RI, and 750 ng of each was run in adjacent slots on 0.8% agarose gels for transfer hybridization. Gel tracks 1–6 contain 0.6, 3, 15, 30, 60, and 150 pg, respectively, of recombinant plasmid DNA that was cleaved with *Pst* I and *Bst* I. The plasmid pRa3.46 (*A*) gives *Pst* I/*Bst* I fragments of 2.5 and 1.6 kb, the latter containing a 0.55-kb cDNA insert. A 0.55-kb sequence present as a single copy within the "unique sequences" of the genome (170,000 kb; ref. 20) represents 2.4 pg of the *Rhynchosciara* DNA loaded onto each track. Similar calculations were made for the clone pRa3.65 (0.61-kb cDNA insert) (*B*) and pRa3.81 (9-kb cDNA insert) (*C*) for which the cDNA sequences correspond to 2.7 and 4 pg, respectively, of single copy *Rhynchosciara* sequences loaded onto each track. (The plasmid pRa3.81 has a *Pst* I site within the cDNA insert that gives rise to an additional 0.7-kb fragment, which is not seen on this gel.) By comparing the autoradiographic response from microdensitometric tracings of the hybridizing band in the *Rhynchosciara* DNA with the known amounts of DNA in the plasmid band, it was possible to calculate the number of copies of the cDNA sequences in *Rhynchosciara* DNA from periods 3 and 6. The sizes (kb) of the fragments from the genomic digest are given by the side of each gel. These were obtained by comparison with the migration of the *Eco*RI fragments of bacteriophage λ DNA, which was mixed in with the *Rhynchosciara* DNA to serve also as a control for the completeness of *Eco*RI digestion.

DISCUSSION

The results in this paper give a more precise quantitation than has been previously possible of the degree of amplification that takes place at DNA puffs in the salivary gland chromosomes of *R. americana* in the late fourth instar. We have found in hybridization experiments with cloned cDNAs from these regions that there is a 16-fold amplification of DNA in both the C3 and C8 DNA puffs. Previous attempts at such measurements have used fractions enriched for rapidly labeled RNA with a high turnover in saturation hybridization experiments (17). Other measurements were based on the kinetics of hybridization with [^{3}H]DNA obtained from the B2 and C3 puffs by microdissection or with sucrose gradient-purified [^{3}H]poly(A)$^+$RNA greatly enriched for sequences hybridizing to the B2 puff (unpublished data). These experiments were severely limited by the degree of purity of the hybridization probe and indicate levels of amplification of usually only 2- to 4-fold.

The levels of gene amplification that we measure at the C3 and C8 puff sites are similar in order of magnitude to those observed for some chorion genes in *Drosophila melanogaster* (25). In both of these cases, we have a developmentally regulated phenomenon aimed at providing particular tissues with large amounts of specific proteins over a short period of time. This contrasts with the gene amplification that is observed in mammalian tissue culture cells in response to metabolic inhibitors (26, 27). Here, higher levels of amplification are achieved by successively culturing cells in increasing concentrations of the drugs. The mechanism for this amplification is not understood, but because in the case of several cell lines it has been possible to observe a repeating pattern in a specific chromosomal region by C-band or Hoeschst 33258 staining (28, 29), it seems likely that large segments of chromosomal DNA are becoming tandemly amplified.

DNA puffing occurs in Sciaridae flies during the last cycle of chromosome polytenization. Autoradiographic studies on the salivary gland chromosomes of *Sciara coprophila* (5), *R. hollanderi* (30), and *R. americana* (31) indicate that DNA synthesis is an ongoing process at most chromosomal regions during this period and suggest that DNA puffing may be related to the general process of polytenization. The incorporation of [^{3}H]thymidine into DNA puffs of *R. americana* is much more effectively inhibited by actinomycin D than is incorporation into other bands that do not amplify and that are simultaneously undergoing DNA synthesis, suggesting that DNA synthesis at the two types of site is subject to a different set of constraints (12). Several attempts have been made to follow the increase in DNA content of the DNA puffs by microspectrophotometry. Rudkin and Corlette (2) detected about a 4-fold increase in the integrated absorbancy of a region corresponding to the B2 puff of *R. angelae* (*americana*), whereas in a region that did not puff, the simultaneous increase was about 2-fold. The measurements of Crouse and Keyl (6) on the DNA puff A on chromosome II of *S. coprophila* showed a 16-fold increase in the integrated absorbancy compared with a 4-fold increase at a neighboring site. In one set of data, a geometrical increase in the DNA content at the puff sites was observed, suggesting that puffing occurs by additional rounds of DNA replication (6). These measurements of a 2- to 4-fold increase in DNA content of the DNA puffs relative to nonpuffed regions are much lower than our measurements from hybridization experiments with cloned

cDNAs. However, microspectrophotometry measures the DNA content over a polytene chromosomal segment that must correspond to a DNA sequence on the order of 100 kb in length. Our measurements, on the other hand, quantitate the amplification of single genes within such a segment. This suggests that the degree of amplification might not be equivalent throughout the puffed region and that spectrophotometry yields an average measurement. This idea is supported by the recent findings of A. C. Spradling (personal communication) that amplification of the chromosomal region 66D in the follicle cells of *D. melanogaster* extends over approximately 100 kb. Not all sequences over this segment are equally amplified; the highest degree of amplification (60-fold) being found in the neighborhood of the structural genes for a number of chorion proteins, including S15 and S18, and decreasing toward the ends of the segment. The same is true of chorion genes in the 7F region, although the maximal amplification here is 12-fold, decreasing to regions where there is no amplification over a 90-kb segment.

It seems probable that Dipteran flies share a common mechanism for gene amplification, perhaps related to their capacity for achieving varying degrees of polyploidy in some of their cells. Sequences such as satellite DNA and rDNA are known to become underrepresented in polytene tissue (32, 33), suggesting that the control of DNA replication in such tissue must differ from that in diploid cells. In the diploid cells of *D. melanogaster* cleavage embryos, replicating chromosomal DNA has been visualized as a serial array of replicated regions created by diverging replication forks. In an elegant study of such DNA, Kriegstein and Hogness (34) were never able to observe the reactivation of replication origins within the daughter segments of such replicated regions. This contrasts with several observations in prokaryotic systems, whereby secondary initiation events from the replication origin are commonly observed before synthesis of the daughter strand is complete. The consequences of permitting reinitiation at some sets of origins in a eukaryotic chromosome but not in others would be the overreplication of the sequences at these regions. Such a model was proposed by Laird *et al.* (35) to explain the structure of polytene chromosomes. We imagine a mechanism for DNA puffing in an extension of these terms: the reinitiation of replication in daughter segments must still be restricted to a large extent in polytene chromosomes in order that the majority of the chromosomal sequences become equally represented, but localized gene amplification would occur if the inhibition of reinitiation were overcome at the puff sites. The ideas of additional rounds of replication at DNA puff sites were apparent in the discussion of Crouse and Keyl (6), but they suggest a model originally proposed by Keyl (36) in which the replicating units are imagined to be circular with fixed ends. This would lead to the tandem reiteration of the replicated region. We favor the reactivation of replication origins within daughter strands, which would lead to various degrees of amplification over a puffed segment. This would explain both the disparity between our data and spectrophotometric measurements on DNA puffs in Sciarid flies and the recent data of Spradling in his studies on what could be a similar phenomenon in *D. melanogaster*. The availability of cloned DNAs from two of the *Rhynchosciara* DNA puffs should now enable us to test these models for DNA puffing.

We thank Mike Scott, Debbie Smith, and Mike Lovett for technical advice; Prof. W. Beermann for his continued support; and Claus Pelling, Cor Hollenberg, Werner Büsen, Orilio Leoncini, and Manuel Diaz for interesting discussions. This work was principally supported by a grant from the Stiftung Volkswagenwerk and also by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo and the Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq). D.M.G. held a fellowship under the exchange program of the Royal Society and the CNPq.

1. Breuer, M. E. & Pavan, C. (1955) *Chromosoma* **7**, 371–386.
2. Rudkin, G. T. & Corlette, S. L. (1957) *Proc. Natl. Acad. Sci. USA* **43**, 964–968.
3. Ficq, A. & Pavan, C. (1957) *Nature (London)* **180**, 983–984.
4. Swift, H. (1962) in *The Molecular Control of Cellular Activity*, ed. Allen, J. M. (McGraw-Hill, New York), pp. 73–125.
5. Gabrusewycz-Garcia, N. (1964) *Chromosoma* **15**, 312–344.
6. Crouse, H. V. & Keyl, H. G. (1968) *Chromosoma* **25**, 357–364.
7. Okretic, M. C., Penoni, J. S. & Lara, F. J. S. (1977) *Arch. Biochem. Biophys.* **178**, 158–165.
8. Bonaldo, M. F., Santelli, R. V. & Lara, F. J. S. (1979) *Cell* **17**, 827–833.
9. Terra, W. R. & de Bianchi, A. G. (1975) *J. Insect Physiol.* **21**, 1547–1550.
10. Winter, C. E., de Bianchi, A. G., Terra, W. R. & Lara, F. J. S. (1977) *J. Insect Physiol.* **23**, 1455–1459.
11. Terra, W. R., de Bianchi, A. G., Gambarini, A. G. & Lara, F. J. S. (1973) *J. Insect Physiol.* **19**, 2097–2106.
12. Berendes, H. D. & Lara, F. J. S. (1975) *Chromosoma* **50**, 259–272.
13. de Moraes, G. W. G. & Miranda, M. (1974) *Cienc. Cult. (São Paulo)* **26**, 403.
14. Winter, C. E., de Bianchi, A. G., Terra, W. R. & Lara, F. J. S. (1977) *Chromosoma* **61**, 193–206.
15. Lara, F. J. S. & Hollaender, F. (1968) *Natl. Cancer Inst. Monogr.* **27**, 235.
16. Winter, C. E., de Bianchi, A. G., Terra, W. R. & Lara, F. J. S. (1980) *Dev. Biol.* **75**, 1–12.
17. Armelin, H. A. & Marques, N. (1972) *Biochemistry* **11**, 3672–3679.
18. Smith, D. F., Searle, P. F. & Williams, J. G. (1979) *Nucleic Acids Res.* **6**, 487–506.
19. Twigg, A. J. & Sherrat, D. (1980) *Nature (London)* **283**, 216–218.
20. Balsamo, J., Hierro, J. M., Birnstiel, M. L. & Lara, F. J. S. (1973) in *Gene Expression and its Regulation*, eds. Kenney, F. T. & Hamkalo, B. A. (Plenum, New York), pp. 101–122.
21. Smith, D. F., McClelland, A., White, B. N., Addison, C. F. & Glover, D. M. (1981) *Cell* **23**, 441–449.
22. Kidd, S. J. & Glover, D. M. (1980) *Cell* **19**, 103–119.
23. Stuart, W. & Porter, D. (1978) *Exp. Cell Res.* **113**, 219–222.
24. Jones, K., Bishop, J. O. & Brito-da-Cunha, A. (1973) *Chromosoma* **43**, 375–390.
25. Spradling, A. C. & Mahowald, A. P. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1096–1100.
26. Alt, F. W., Kellems, R. E., Bertino, J. R. & Schimke, R. T. (1978) *J. Biol. Chem.* **253**, 1357–1370.
27. Wahl, G. M., Padgett, R. A. & Stark, G. R. (1979) *J. Biol. Chem.* **254**, 8679–8689.
28. Dolnick, B., Berenson, R., Bertino, J. R., Kaufmann, R. J., Numberg, J. H. & Schimke, R. T. (1979) *J. Cell. Biol.* **83**, 394–402.
29. Bostock, C. J. & Clark, E. M. (1980) *Cell* **19**, 709–715.
30. Simoes, L. C. G. (1970) *Rev. Bras. Biol.* **30** (2), 191–199.
31. Machado-Santelli, G. M. & Basile, R. (1974) *Cienc. Cult. (São Paulo)* **27**, 167–174.
32. Gall, J. G., Cohen, E. M. & Polan, M. L. (1971) *Chromosoma* **33**, 319–327.
33. Gambarini, A. G. & Lara, F. J. S. (1974) *J. Cell Biol.* **62**, 215–222.
34. Kriegstein, H. J. & Hogness, D. S. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 135–139.
35. Laird, C. D., Chooi, W. Y., Cohen, E. M., Dickson, E., Hutchinson, N. & Turner, S. H. (1973) *Cold Spring Harbor Symp. Quant. Biol.* **38**, 311–328.
36. Keyl, H. G. (1965) *Experientia* **21**, 191–193.