Gene amplification in *Rhynchosciara* salivary gland chromosomes
(cDNA clones/DNA puffs/C chromosome)

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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* polynel 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 [3H]thymidine (3). Subsequently, similar observations were made on the salivary chromosomes of larvae from the genus Scaara (4–6).

The DNA puffs, which appear late in fourth instar in *Rhynchosciara* salivary glands, are involved in the production of messenger RNAs (7, 8). These encode several peptides of the commumal cocon, which are needec in large amounts over a short period of time (9, 10). Based on morphological and physiologival 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staining band between two orcin (+) bands at the chromosomal sites of these puffs early in period 4 (ref. 12; unpublished data).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)+ 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).

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.

Abbreviation: kb, kilobase(s).

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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 EcoRI-cleaved pAT153 (19), which had been tagged 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 32P to specific activities greater than 10^9 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 [32P]DNA complementary to RNA from period 5 glands but that would not hybridize with [32P]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 giv-
ing an intense autoradiographic signal, to select plasmids com-
plementary 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 exper-
iment, 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 spe-
cies 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 pRa3.91,
which is complementary to the 1.95-kb RNA. We localized
these sequences on the R. americana salivary gland chromo-
somes 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 EcoRI-cleaved salivary
gland DNA from period 3 (designated Ra³) before puffing oc-
curs and from period 6, when the C3 DNA puff reaches its
maximum size (designated Ra⁶). The autoradiographic re-
sponse 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 en-
codes the pRa3.46 sequences (Fig. 4A) is contained within a 16-
kb EcoRI 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. 4B 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 ex-
periment with the plasmids pRa1.66 and pRa6.85, which con-
tain 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. 4C 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 hybrid-
ized to a 3-kb EcoRI fragment encoding a sequence that am-
plified 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 EcoRI frag-
ment but also to a 6.2-kb fragment. In this experiment we cal-
culated a 16-fold amplification of the coding sequences in each
of these fragments (data not shown).

FIG. 3. In situ hybridization of pRa3.65. [³H]RNA synthesized
from pRa3.65 was hybridized with period 6 salivary gland chromo-
somes, and the slides were exposed for 1 wk. The main site of hy-
bridization at the C3 puff (C3d) is indicated by the arrow. There are se-
veral 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) An-
other set of chromosomes hybridized with cRNA from pRa3.65, in
which C3 is preferentially labeled.
**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 4th 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 $[^3H]$DNA obtained from the B2 and C3 puffs by microdissection or with sucrose gradient-purified $[^3H]$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 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 choriogen 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 mamalian 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 Hoescht 33258 staining (28, 29), it seems likely that large segments of chromosomal DNA are becoming tandemly amplified.

DNA puffing occurs in Sciaraid 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 $[^3H]$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 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 sequences.
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 polyplody 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 over-replication 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 idea 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 also dispart 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.

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