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Genetic and Physical Linkage of Exogenous Sequences in Transformed Cells

Manuel Perucho, Douglas Hanahan* and Michael Wigler
Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724
*Department of Biochemistry and Molecular Biology
Harvard University
Cambridge, MA 02138

Summary

The calcium phosphate precipitation method of Graham and van der Eb (1973) is an efficient means of introducing DNA into cultured animal cells. Cells which incorporate one selectable marker are also likely to incorporate sequences from the carrier DNA. Both selected and unselected markers are found integrated in the high molecular weight nuclear DNA of the host. In the present study, we demonstrate that exogenously acquired sequences are gentically linked, segregating and amplifying coordinately, and that their flanking sequences derive primarily from the carrier species rather than the host species. Based on these results, we propose that, upon transformation, the host cell ligates incorporated DNA into a large concatameric structure which may at times be as large as 2000 kilobases. From blotting data alone we cannot determine whether this structure is chromosomal or extrachromosomal in location.

Introduction

Methods for the stable introduction of purified genes into cultured cells offer new approaches for the study of gene expression. Informed use of these methods requires knowledge of the physical state of DNA incorporated into the new host genome. The most efficient method of gene transfer currently in common use is the method developed by Graham and van der Eb (1973), in which DNA is co-precipitated with calcium phosphate and the precipitate is added directly to a cell monolayer. In this way cells can be transformed with selectable markers of viral (Graham et al., 1974; Bacchetti and Graham, 1977; Maitland and McDougall, 1977; Wigler et al., 1977) or cellular origin (Wigler et al., 1978, 1979a; Graf, Urlarb and Chasin, 1979; Willecke et al., 1979). Efficient transformation requires the addition of carrier DNA as a component of the precipitate (Graham and van der Eb, 1973). Cells which take up a selectable marker often incorporate many other sequences present in the carrier (Wigler et al., 1979b). In this way, or by direct ligation to a selectable marker, it is possible to transform cells with unselectable genes (Mantei, Boll and Weissman, 1979; Wold et al., 1979; Breathnach, Mantei and Chambon, 1980; Lai et al., 1980). Under selective pressure, exogenous sequences are integrated in a stable fashion within the high molecular weight nuclear DNA of the host cell (Pellicer et al., 1978; Wigler et al., 1979b). In this manuscript we present evidence that exogenous sequences incorporated by the host are ligated together into a new genetic unit which may sometimes be as large as 2000 kb.

Results

Early Stabilization of Co-transformed Genotype

In a previous report (Wigler et al., 1979b) it was shown that co-transformed DNA sequences present in mouse cells were stable for many generations in culture. Different subclones of established lines, derived by transformation of Ltk$^-$ cells with the herpes simplex virus (HSV-1) thymidine kinase (tk) gene, showed virtually identical annealing patterns for co-transformed DNA sequences. We have now extended these observations and have demonstrated that the stabilization of the co-transformed genotype is, in many cases, an early event. Ltk$^-$ cells were transformed with 1 ng of the HSV-1 tk gene and co-transformed with 1.0 μg of Pst I-cleaved bacteriophage ϕX174RF DNA and 20 μg of carrier salmon DNA per dish. After selection in HAT medium, eight colonies were picked at an early stage (8–20 cells per colony). The individual cells of each colony were subcloned into HAT in Multiwell dishes and then grown into mass culture (approximately 35 cell generations). DNA was prepared from each of the subclones, digested with the restriction endonuclease Hind III and analyzed by filter hybridization for the presence of ϕX174 sequences. Two of the initial eight clones contained no detectable phage sequences in any subclones. Six of the remaining clones showed distinct annealing patterns but the annealing patterns of all sibling subclones were identical. This is illustrated for two sets of subclones in Figure 1: ten subclones from LtϕC (lanes A–J) and three subclones from LtϕD (lanes K–M). These results indicate that the stable arrangement of exogenous sequences in the transformed host occurs at the same time as or soon after the acquisition of the transformed phenotype.

Genetic Linkage of Co-transforming Elements

Although the arrangement of exogenous sequences in tk$^+$ transformants is stable while selective pressure is maintained, their stability in tk$^-$ revertants has not been characterized previously. To examine the retention of co-transformed sequences in revertants of different tk$^+$ transformants, revertant subclones were obtained by selection in medium containing BUdR (Kit et al., 1963).

One tk$^+$-transformed line which we have studied in great detail is ϕX-4. ϕX-4 is an HSV tk$^+$ Ltk$^-$ derivative containing approximately 25 integrated copies of bac-

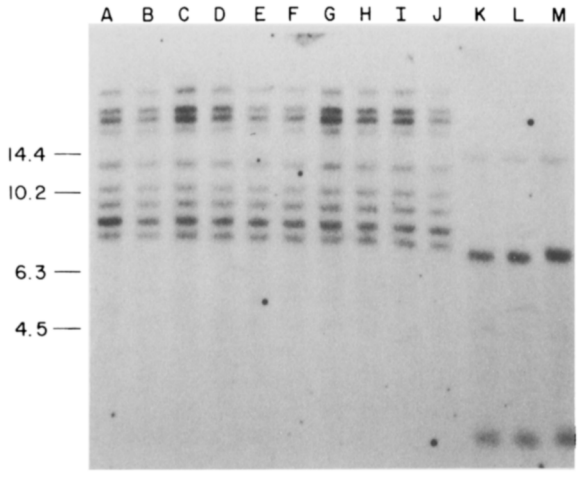

Figure 1. Annealing Pattern of Co-transformed Sequences in Sibling Subclones of HSV-1 tk^+ Ltk^- Transformants

High molecular weight DNA was isolated from ten sibling subclones of LϕPC (lanes A–J) and three sibling subclones of LϕPE (lanes K–M). 10 μg of each DNA were cleaved with Hind III, electrophoresed in 0.5% agarose gels and analyzed by filter hybridization using ^{32}P-labeled nick-translated ϕX174 (3×10^6 cpm/μg) as probe. The numbers at the left indicate molecular weights (in kb) of restriction fragments of adenovirus 2 DNA.

teriophage ϕX174RF DNA (Wigler et al., 1979b). ϕX174 sequences can be visualized as discrete bands by blot hybridization after digestion of host DNA with restriction endonucleases which do not cleave within the bacteriophage genome. tk^+ subclones of ϕX-4 display the same annealing profile as the parental line (Wigler et al., 1979b; and data not shown). These cells plate in BUdR with 0.1% the efficiency of plating in HAT. Twenty different BUdR-resistant subclones were isolated and grown into mass culture (about 25 cell generations). All BUdR-resistant subclones failed to grow in HAT medium (plating efficiencies $<10^{-6}$). When analyzed by blot hybridization, revertant subclones had no detectable sequences homologous to the HSV tk gene (Figure 2b, lanes E–L), while four parallel subclones grown in HAT contained an identically migrating homologous band (Figure 2b, lanes A–D). These results indicate that revertants selected in BUdR arise by loss of the tk gene. To examine loss of co-transformed sequences, DNA from each revertant subclone was digested with Hind III and the size and number of fragments containing ϕX174 sequences were compared to the parental line. Eight revertants lost all traces of ϕX174 sequences. The remaining twelve each lost some of the sequences formerly present. tk^+ subclones made at the same time show a blot pattern indistinguishable from that of the ϕX-4 parent (data not shown). Blot hybridization analysis of representative revertant subclones is shown in Figure 2a (lanes C–L). The parental pattern is shown in lanes B and M. As shown in the figure, loss of particular integrated sequences of ϕX174 is not random: some sequences have a higher probability of retention than others.

We have analyzed several other transformants in the same manner. Figure 3 is an analysis of poTC10, which was obtained by transformation of Ltk^- cells with the HSV-1 tk gene and co-transformation with Sal I-cleaved poT, a bacterial plasmid containing the entire early region of SV40 (Hanahan et al., 1980). HAT-selected subclones (lanes A–C) show a similar pattern of annealing for the co-transformed sequences, while tk^- revertants have lost some or all of the poT sequences (lanes D–K). Figure 4 illustrates the results using another recipient cell, 143, a tk^- human osteosarcoma cell line (Bacchetti and Graham, 1977). This line was transformed with the cloned HSV-1 tk gene and co-transformed with the Eco RI-A restriction fragment of adenovirus 2 DNA. Two transformants, HosII a2 and HosII a5, were identified which contained a single copy of adenovirus 2 sequences (Grodzicker and Klessig, 1980). DNAs from different subclones were analyzed by filter hybridization for the presence of co-transformed sequences. The adenovirus 2 sequences were stable in cells subcloned under selective pressure (lanes B, C). Revertants obtained by selection in BUdR, however, lost these sequences (lanes D, E and G–K). One revertant from the line HosII a2 still contained some adenovirus sequences but the size of the hybridizing restriction fragment was smaller (lane L) than the fragment present in the parental line (lane F). Blot analysis of the HSV tk gene sequence content showed that all the tk revertants arose by loss of the tk gene sequences (data not shown).

To study this process using another selectable marker, Ltk^- cells were transformed with the chicken tk (chtk) gene using total chicken genomic DNA as donor and were co-transformed with ϕX174RF DNA. One transformant, CϕX-3, which contained five discrete bands homologous to ϕX174 DNA, was chosen for further study. Subclones of the line CϕX-3 maintained in the selective medium (HAT) showed a stable arrangement of co-transforming sequences (Figure 5a, lanes B–D), whereas tk^- revertants selected by subcloning in BUdR lost some (lane E) or all (lanes F–J) of the ϕX174 sequences. Blot analysis of the tk gene content of CϕX-3 subclones using purified chicken tk gene as probe (Perucho et al., 1980) showed the existence of homologous bands to the chicken tk gene in cells growing in HAT (Figure 5b, lanes B–D). No hybridizing bands were detected in the revertants (Figure 5b, lanes E–J), indicating that the reversion arose by loss of the transforming chtk gene.

These examples and others not presented illustrate the consistent pattern of loss of co-transforming sequences upon reversion of the selected phenotype. This pattern of loss is compatible with two propositions: reversion occurs by loss or deletion of the

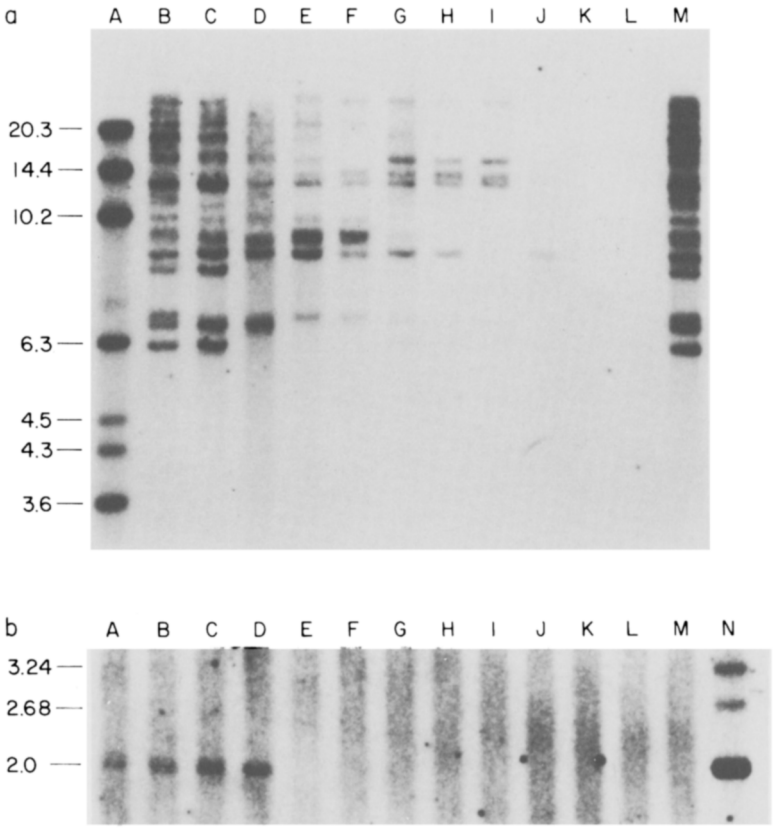

Figure 2. Annealing Pattern of Co-transformed φX174 Sequences in tk⁻ Revertants of φX-4

(a) tk⁻ revertants from the line φX-4 were isolated by subcloning in medium containing BUdR, and high molecular weight DNA was prepared from each of these subclones. 10 μg of each DNA were cleaved with Hind III, electrophoresed in a 0.8% agarose slab gel and analyzed by blot hybridization using ^{32}P-labeled nick-translated φX174 DNA (4×10^8 cpm/μg) as probe. (Lane A) Mixture of Eco RI and Bam HI digests of ^{32}P-labeled adenovirus 2 DNA. The molecular weights (in kb) of these fragments are at the left. (Lanes B and M) φX-4 parental line. (Lanes C–L) Ten different subclones resistant to BUdR.

(b) 10 μg of DNA from subclones in HAT (lanes A–D) or BUdR (lanes E–L) from the line φX-4 were digested with Pvu II and electrophoresed through 0.8% agarose gels. Blot analysis was performed using as probe the nick-translated, purified 3.4 kb Bam HI fragment of ptk2 containing the HSV-1 tk gene (1×10^8 cpm per μg) (Hanahan et al., 1980). (Lane M) 10 μg of Ltk⁻ DNA digested with Pvu II; (lane N) 60 pg of Pvu II-cleaved ptk2. The lines at the left indicate the molecular weights of these ptk2 fragments in kb.

transforming gene and the size of deletion may vary from revertant to revertant; and co-transforming sequences are arrayed about the regions flanking the transforming gene. From these propositions it follows that large deletions will result in the loss of all co-transformed sequences, while small deletions will result in partial loss: the co-transforming sequences most distant from the transforming gene would be retained after a small deletion.

Co-amplification of Co-transformed Sequences

Genetic linkage between co-transformed markers can also be demonstrated using an amplifiable vector. Wild-type cultured cells are sensitive to the folic acid analog methotrexate (mtx), a specific inhibitor of dihydrofolate reductase (dhfr). Wild-type cells can be rendered resistant to low levels of mtx by transformation (Lewis et al., 1980; Wigler et al., 1980) using total genomic DNA from A29 cells, a CHO derivative encoding a mutant dhfr with lowered affinity for mtx (Flintoff, Davidson and Siminovitch, 1976). When initial transformants are exposed to still higher concentration of mtx, a resistant population emerges which has an increased dosage of the exogenously acquired dhfr gene (Wigler et al., 1980). Gene amplification as a mechanism of resistance to selective pressure was first demonstrated by Schimke and colleagues for the endogenous dhfr gene of mouse (Schimke et al., 1978). The size of the amplification unit is not known, but in the mouse it must exceed 40 kb, the minimum size estimate of the dhfr gene itself (Nunberg et al., 1980). If the amplification unit extends well beyond the gene into its flanking sequences, these sequences would be amplified as well during selection. We therefore tested whether sequences co-transformed into mouse cells using the hamster A29 dhfr gene as a selectable marker could be amplified by selecting cells resistant to increased levels of mtx.

Ltk⁻ cells were transformed with A29 genomic DNA and co-transformed with T18g, a derivative of the E. coli plasmid pBR322 containing the yeast Leu 2 gene and a serine tRNA suppressor. Several transformed clones were grown into mass culture and populations resistant to 0.1, 2.0 and 10.0 μg/ml mtx were derived. DNAs from these cultures were purified and analyzed for the presence of T18g sequences by blot hybridization. Four independent transformants initially resistant to 0.1 μg/ml mtx were examined in this manner (Figure 6). DNAs were cleaved with restriction endonuclease Xba I, which does not cleave T18g. Each line contained T18g sequences in a unique blotting pattern. T18g-specific bands of two lines, Ib1 and Ib2, did not increase in intensity as cells resistant to high levels of mtx were selected. The sequence content of two other transformants, Ib3 and Ib6, apparently increased as cells resistant to 2.0 and 10.0 μg/ml mtx were selected. In the line Ib3, one of two hybridizing

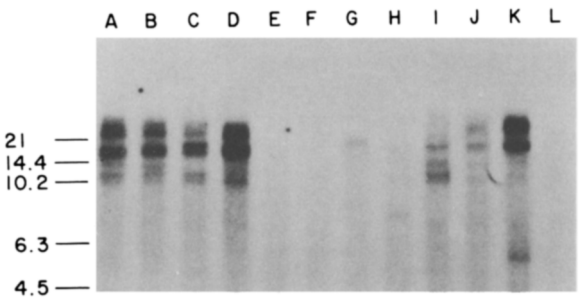

Figure 3. Annealing Pattern of poT Sequences in Co-transformants and tk⁻ Revertants

High molecular weight DNA was extracted from different subclones of the line poTC10, cleaved with Xba I and analyzed by filter hybridization after electrophoresis through a 0.8% agarose gel, using nick-translated poT DNA (2×10^8 cpm per μg) as probe (Hanahan et al., 1980). (Lanes A–C) Three different subclones in HAT medium; (lanes D–K) eight different subclones selected in BUdR; (lane L) Ltk⁻ DNA digested with Xba I. The lines indicate the position and molecular weight (in kb) of adenovirus 2 DNA restriction fragments.

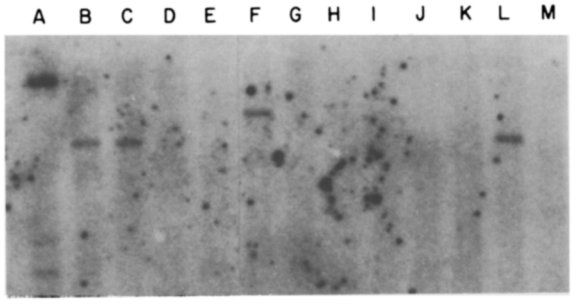

Figure 4. Hybridization Profile of Adenovirus Sequences in Co-transformants of 143 and tk⁻ Revertants

Subclones of the lines HosII a5 (lanes B–E) and HosII a2 (lanes F–L) were isolated in either HAT (lanes B, C and F) or BUdR (lanes D, E, G–L). DNA was extracted, digested (10 μg each) with Eco RI and electrophoresed through 0.8% agarose gels. Blot hybridization was performed using nick-translated adenovirus 2 DNA as probe (7.4×10^7 cpm/μg). (Lane A) 100 pg of adenovirus 2 DNA digested with Eco RI, mixed with 10 μg of salmon DNA; (lane M) 10 μg of 143 recipient cell DNA, digested with Eco RI.

bands increased in intensity, while in Ib6, all bands present increased in intensity during amplification (not all bands present are visible in this blot). These results imply that at least some of the co-transformed sequences have integrated within the amplification unit of the exogenous dhfr gene.

Plasmid Rescue of Co-transformed Sequences

The previous results have demonstrated the genetic linkage of transforming elements. Two plausible models of linkage can be imagined. In the first, exogenous sequences become linked together by the cell during the transformation process into a large concatameric structure. In the second, exogenous sequences become integrated independently into one host chromosome or one region of a chromosome. If the first model is correct, the DNA flanking an exogenous sequence should in most cases derive from the carrier species. If the second model is correct, flanking DNA will derive from the host species. To choose between these models we co-transformed Ltk⁻ cells with pBR322 derivatives and then used the plasmid sequences residing in the transformants to "rescue" the flanking animal sequences into E. coli. This approach has been described previously (Hanahan et al., 1980; Perucho et al., 1980). Genomic DNA from the transformants was digested with restriction endonucleases. Cleaved DNA was then ligated under cyclization conditions, and cyclized DNA was used to transform E. coli to ampicillin resistance.

Three plasmids (pC10S, plb3R and plb6R) were rescued from three different transformants. pC10S was rescued from poTC10 (see Figure 3), a line containing the pOT plasmid (Hanahan et al., 1980). Salmon DNA was used as carrier when poTC10 was created. plb3R and plb6R were rescued from Ib3 and Ib6, respectively (see Figure 6), two mtx-resistant Ltk⁻ derivatives transformed with the hamster dhfr gene encoded by A29 cells, and co-transformed with the T18g plasmid. pC10S was rescued by cleavage of host DNA with Sst I, and plb3R and plb6R were rescued by Xba I cleavage of DNA from dhfr-amplified lines resistant to 10 μg/ml mtx.

To verify that these plasmids were indeed rescuants rather than chance contaminants, they were nick-translated and used as probes in blot hybridization experiments (see Figure 7). In Figure 7c, lanes A and B show that Sst I-cleaved pC10S co-migrated with a homologous Sst I fragment of poTC10 cellular DNA. Figure 7b demonstrates that plb6R linearized by cleavage with the rescuing enzyme Xba I (lane D) co-migrated with a homologous Xba I fragment of Ib6 genomic DNA (lane C). Double digestion with Eco RI and Xba I gave rise to two fragments (lane F) which co-migrated with homologous fragments present in the host DNA (lane E). There are other bands present in Ib6 genomic DNA which presumably derived from other amplified co-transformed sequences that we did not rescue. plb6R hybridized extensively with yeast DNA (lane B), indicating the derivation of plb6R from the original T18g co-transforming plasmid. A similar analysis of plb3R confirmed its derivation from T18g by way of co-transformation and rescue from Ib3 cells (see Figure 7a). From the intensity of the hybridizing bands in the parental lines, we infer that plb3R was rescued from an unamplified T18g sequence, whereas plb6R was derived from an amplified one. From detailed restriction maps of plb3R and plb6R we have reconstructed the events that led to the rescue of these plasmids from co-transformed cells (Figure 8). A similar reconstruction for the pC10S rescuant has been described previously (Hanahan et al., 1980).

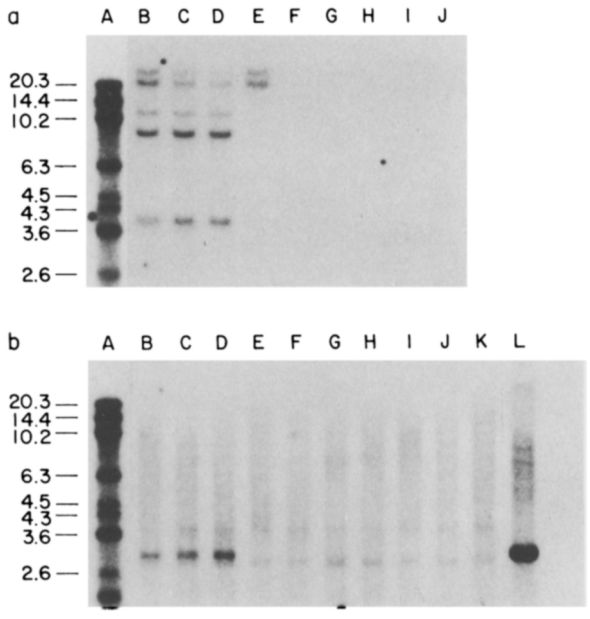

Figure 5. Hybridization Profile of ϕX174 Sequences in Co-transformants and Revertants of Chicken tk^+ Ltk^-

High molecular weight DNA from three independently isolated subclones in HAT (lanes B–D) and six subclones in BUdR (lanes E–J) from the line CϕX-3 were digested (10 μg each) with either Bam HI (a) or Hind III (b) and electrophoresed through 0.8% agarose gels. Blot analyses were performed using ^{32}P-labeled nick-translated ϕX174 DNA (a) or purified Xba I/Kpn fragment of pchtk2 (b) (Perucho et al., 1980), comprising chicken tk sequences, as probes (2 and 1 $\times$ 10^6 cpm per μg, respectively). (Lane A) Mixture of Eco RI and Bam HI digests of ^{32}P-labeled adenovirus 2 DNA. The lines indicate the molecular weights (in kb) of these adenovirus fragments. (Lanes K and L) 10 μg of Hind III digests of Ltk^- and chicken DNA, respectively.

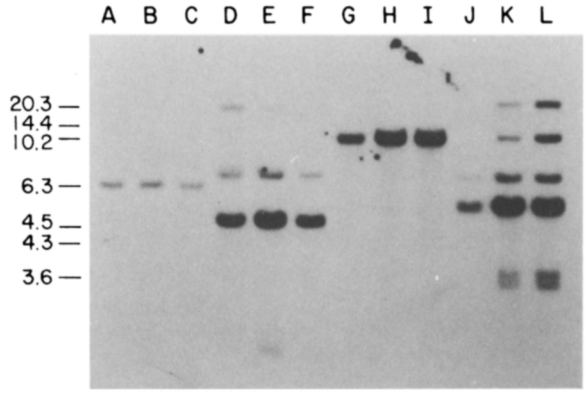

Figure 6. Amplification of Co-transformed T18g Sequences

High molecular weight DNA was extracted from several different clones of mtx-resistant Ltk^- cells which had been co-transformed with T18g plasmid. 10 μg of each DNA were digested with Hind III, electrophoresed through 0.8% agarose gels and analyzed for the presence of T18g sequences by filter hybridization using ^{32}P-labeled nick-translated T18g DNA as probe (2 $\times$ 10^8 cpm per μg). (Lanes A–L) DNA from cell lines Ib1 (A, B and C), Ib2 (D, E and F), Ib3 (G, H and I) and Ib6 (J, K and L), grown in 0.1 (lanes A, D, G and J), 2.0 (lanes B, E, H and K) and 10 (lanes C, F, I and L) μg/ml of mtx. The lines at the left indicate the position and molecular weights of restriction fragments of adenovirus 2 DNA. By ethidium bromide staining of the gel, all cellular DNAs were present in approximately equal amounts.

Flanking Sequences of Co-transforming Elements Derive from Carrier

To identify the origin of the sequences which flank the co-transformed T18g sequences, we nick-translated the rescuants and used them as probes in blot hybridization experiments (Figure 9). Radioactive probes derived from pC10S (Figure 9c) and plb6R (Figure 9b) hybridized intensely and diffusely with restriction endonuclease-cleaved DNA from the species used as carrier but not with host mouse DNA. Under the hybridization conditions used, annealing to unique sequence DNA would be visible but might be masked by hybridization due to a component in the probe annealing to repetitive DNA. Nick-translated plb3R (Figure 9a) also hybridized intensely and diffusely to its carrier species (hamster), although faint bands were observed with mouse DNA when hybridization was performed at 65°C (lane C). When the stringency for hybridization was increased by raising the temperature to 68°C (lanes D–F) and 71°C (lanes G–I), the hybridization to mouse sequences disappeared and hybridization to specific hamster fragments became visible against a less intense background of diffuse hybridization. These experiments provide evidence that the rescued plasmids contain sequences derived from the carrier rather than the host species.

More direct evidence for this conclusion comes from solution hybridizations experiments. Restriction endonuclease fragments of the rescuants were purified which contained primarily or exclusively host flanking sequences. These were nick-translated and used as probes in annealing experiments with vast excesses of genomic DNA from various species. Annealing was measured by S1 protection. The kinetics of annealing of flanking sequences derived from pC10S in the presence of salmon, mouse or hamster DNA are shown in Figure 10. The accelerated annealing of these sequences indicates that they derive from a highly repetitive component of salmon DNA. A similar study of plb6R indicated that the flanking sequences derived from hamster DNA.

Discussion

We have examined the physical state of exogenous sequences within cells transformed using the calcium phosphate technique of Graham and van der Eb (1973). Our data indicate that exogenous sequences within transformed hosts are genetically linked, segregating and amplifying coordinately, and that their flanking sequences derive from the carrier species rather than from the host species. The simplest model consistent with these findings is that exogenous DNA

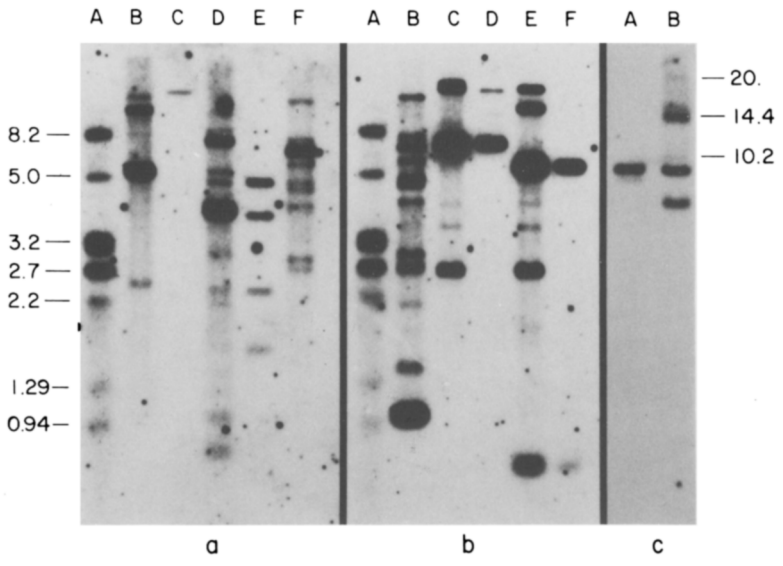

Figure 7. Analysis of the Origin of Rescued Plasmids by Blot Hybridization

plb3R (a), plb6R (b) and pC10S (c) plasmids were ^{32}P-labeled by nick translation (2×10^8 cpm per μg) and used as probes in hybridization blots to different DNAs electrophoresed through 1.0% (a and b) and 0.7% (c) agarose gels, respectively.

(a) (Lanes B and D) 10 μg each of DNA from lb3 cleaved with Xba I and Xba I/Eco RI, respectively; (lanes C and E) 10 and 50 pg of Xba I and Xba I/Eco RI-cleaved plb3R, respectively, with 5 μg of carrier Ltk$^-$ DNA; (lane F) 10 μg of Bam HI/Hind III-digested yeast DNA.

(b) (Lanes C–F) Xba I (C and D) and Xba I/Bam HI (E and F) digests of lb6 (C and E) DNA (10 μg each) or plb6R (D and F) (100 pg each with 5 μg of carrier Ltk$^-$ DNA), respectively; (lane B) 10 μg of yeast DNA digested with Bam HI/Hind III. Lane A in (a) and (b) is a Hind III digest of ^{32}P-labeled adenovirus 2 DNA. The molecular weights of the main restriction fragments are indicated at the left in kb.

(c) (Lane A) 50 pg of pC10S plasmid digested with Sst I, with 5 μg of carrier chicken DNA; (lane B) 10 μg of poTC10 DNA digested with Sst I. The lines at the right indicate the position and molecular weights (in kb) of adenovirus 2 DNA restriction fragments.

is incorporated by the cell into a large concatameric structure by a process of intermolecular ligation.[1] *Intra*molecular ligation during transfection with linearized SV40 has been described by Lai and Nathans (1974). Since we find that all sequences that are stably retained in a transformant population are genetically linked to the selectable marker, we conclude that each cell usually contains only one concatameric "package" of exogenous DNA and that concatamerization is a far more rapid event than chromosomal integration. (We have, however, noted in our unpublished work several instances in which cells selected for the transfer of both tk and aprt appear to have acquired two independently segregating genetic units.) The stable arrangement of co-transformed sequences appears to occur quite early in the lineage of a transformed clone. It seems plausible that other sequences may be transiently incorporated by cells but subsequently lost when they are not integrated into the evolving concatamer of exogenous DNA and thereby stabilized by linkage to the selectable element. Work in progress supports this notion.

The size of the transforming "package" in the cell line ϕX-4 can be estimated as follows. The line contains approximately 25 copies of the 4 kb ϕX174 genome which was present at one part to twenty of the carrier during the transformation event. If carrier sequences were incorporated with the same efficiency on a mass basis as ϕX174 sequences, ϕX-4 would contain $25 \times 4 \times 20$ or 2000 kb of carrier sequence. In general, there is considerable variation in the amount of carrier incorporated by a transformant as judged by variation in the number of copies of marker sequences incorporated. Other methods of estimating this size, based on the co-transformation frequencies of unlinked markers, suggest that the average is in the range of 1000 kb (work in progress).

It is unclear whether exogenous DNA is integrated into a host chromosome or replicates independently as an extrachromosomal unit. Some transformants are genetically stable in the absence of selective pressure, while others not (Wigler et al., 1979a). The former class may represent instances in which the exogenous DNA becomes chromosomally linked. In at least one instance, exogenous sequences have been shown to be chromosomally linked (Smiley et al., 1978). The genetically unstable transformants may represent instances in which the foreign sequences replicate extrachromosomally and are lost with high frequency through asymmetric segregation during mitosis. Other explanations of highly unstable genotypes are also possible.

Although it appears from our studies of genetic linkage that concatamer formation is part of the major pathway of co-transformation, other pathways for transformation are possible. For example, we have examined only transformants derived from cell populations exposed to DNA mixtures in which the selectable marker has comprised a very minor proportion. This may bias transformation to a particular pathway in a subpopulation of cells. Evidence for the chromosomal integration of a transforming gene comes from

[1] "Pekelasome" is the name we have previously proposed for the theoretically predicted structure consisting of concatamerized exogenously acquired DNA, residing either extrachromosomally or integrated as a unit in host chromosomal DNA.

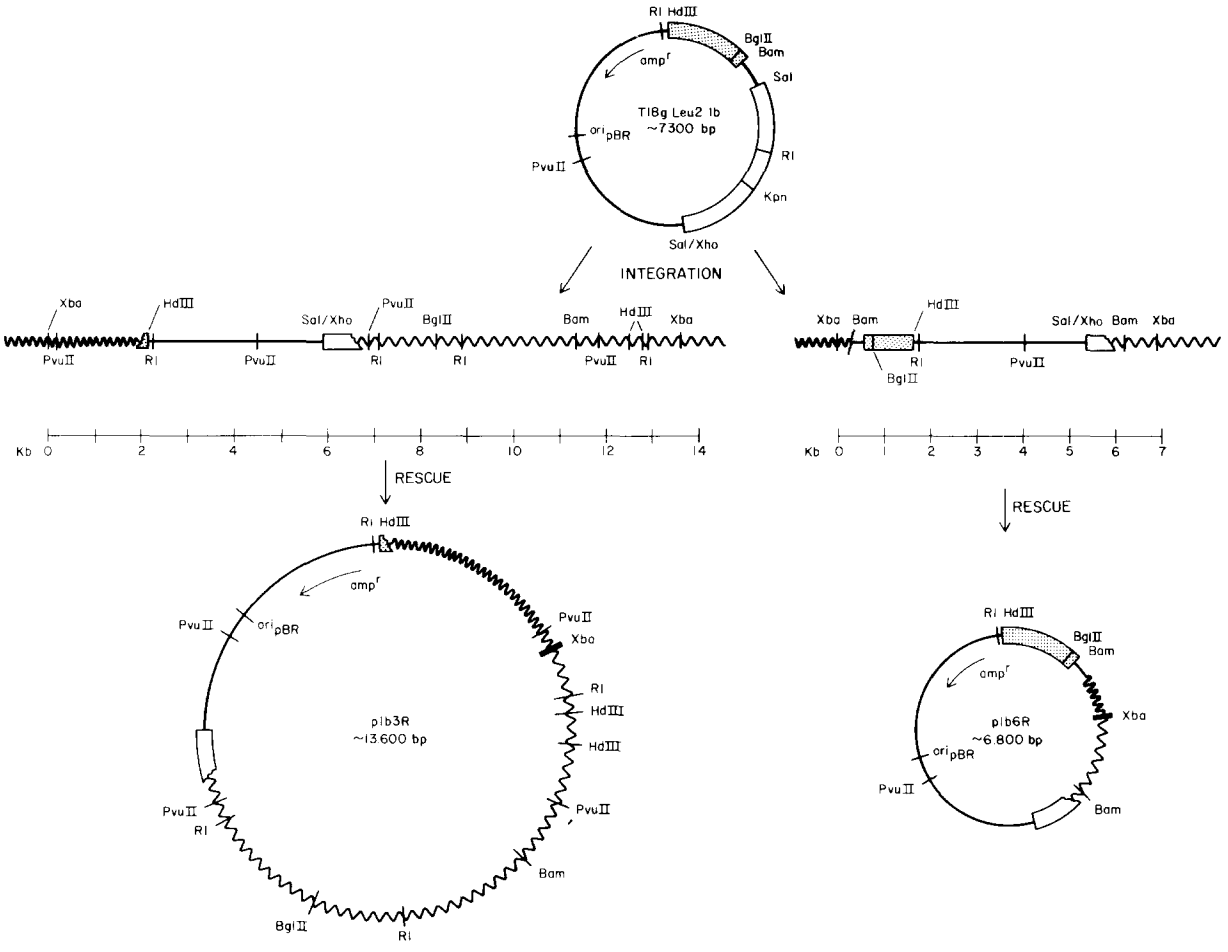

Figure 8. Schematic Representation of the Integration of T18g in Co-transformants and Rescue of plb3R and plb6R

Ltk$^-$ aprt$^-$ cells were transformed with genomic DNA (coding for mtx resistance) from the hamster A29 cell line, and co-transformed with T18g circular plasmid. plb3R and plb6R were rescued by cleavage of transformants with Xba I and ligation of DNA from lb3 (left) and lb6 (right). All the sites indicated in the restriction maps of plb3R and plb6R were verified by restriction analysis. Left and right flanking sequences of the co-transformed plasmids are indicated by (▲▲) and (〰), respectively.

the work of Botchan et al. (1980), who examined the sequences flanking an SV40 integrant in a rat cell transformed with Eco RI-linearized SV40 DNA. In that instance, calf thymus DNA had been used as carrier (M. Botchan and J. Sambrook, personal communications), yet one end of the SV40 integrant was shown to be embedded in rat sequences.

The model we propose has some interesting implications. First of all, the local sequence environment of an unselected marker will derive from the carrier species rather than from the host. Second, this sequence environment may be disorganized in some fundamental ways, being a patchwork construction of chromosomal fragments rather than a naturally evolved chromosomal region. Thus the environment of the transforming sequences may consist of random linkages of satellite sequences, ribosomal genes, or other repetitive sequences intermingled with unique copy DNA all derived from the carrier DNA. This disorganized environment may have profound consequences for gene expression and sequence stability. With respect to the latter point we note that the reversion frequency of transformants is in fact quite high (Wigler et al., 1977), and for the transformants analyzed in this paper, particularly ϕX-4 (10^{-3}) and HosII a2 (10^{-1}), reversion is not always due to complete loss of all foreign sequences. Rather there is a high frequency of partial deletions. We do not know at present if this reflects the scrambled or repeated structure of the sequence environment of the transforming element, its cellular location or normal levels of a constitutive cellular activity. Third, recombinational events are likely to occur between genes in the carrier, establishing new linkage arrangements and creating new chimeric genes. It may be possible to avoid some of these complexities by transforming cells in the absence of carrier. Transformation is much less efficient, however, when carrier is omitted. Other

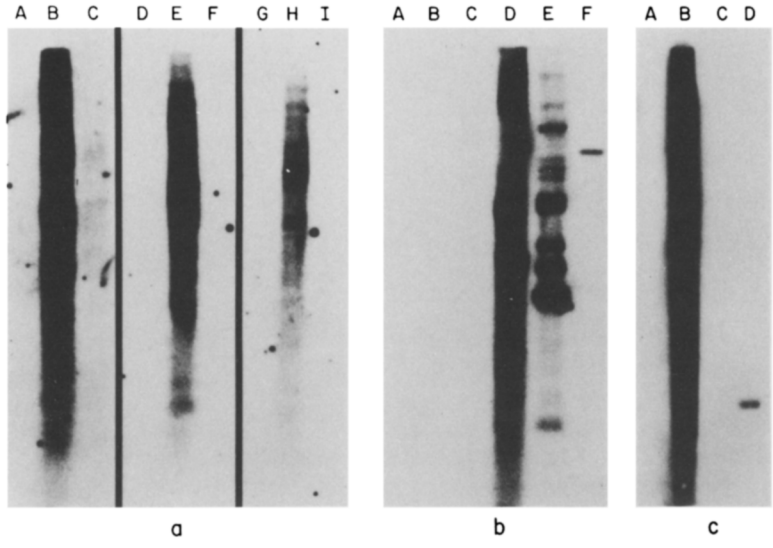

Figure 9. Blot Analysis of the Rescued Plasmids

The rescued plasmids plb3R (a), plb6R (b) and pC10S (c) were ^{32}P-labeled by nick translation and used as probes in hybridization blots to DNA from various species electrophoresed through 1% agarose gels.

(a) DNAs from salmon (lanes A, D and G), hamster (lanes B, E and H) and mouse (lanes C, F and I) digested with Xba I/Eco RI (10 μg each). The hybridizations were carried out in 6× SSC for 24 hr at 65°C (lanes A, B and C), 68°C (lanes D, E and F) and 71°C (lanes G, H and I).

(b) (Lanes A–E) DNAs from chicken, salmon, mouse, hamster and yeast, respectively, digested with Xba I/Bam HI (10 μg each); (lane F) plb6R digested with Xba I (50 pg with 5 μg of chicken carrier DNA).

(c) (Lanes A, B and C) DNAs from chicken, salmon and mouse, respectively, digested with Sst I/Bam HI (10 μg each); (lane D) the purified 1.2 kb Sst I/Bam HI fragment of pC10S (10 pg) with 5 μg of chicken carrier DNA.

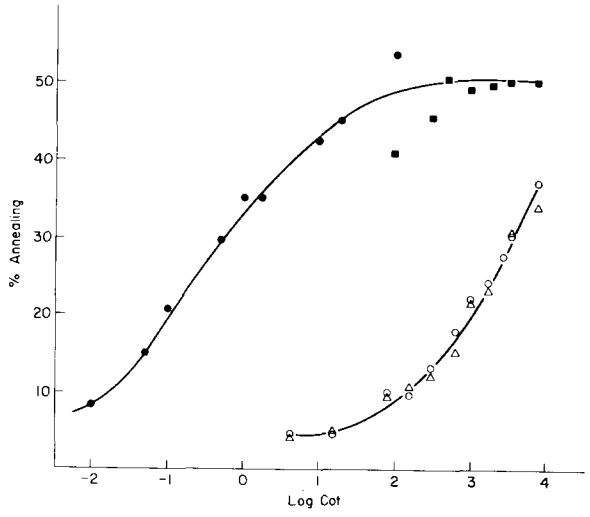

Figure 10. Kinetics of Hybridization of the 1.2 kb Bam HI/Sst I Fragment of pC10S to Mouse, Hamster and Salmon DNAs

The Sst I/Bam HI 1.2 kb fragment of pC10S comprising flanking sequences (Hanahan et al., 1980) was purified by agarose gel electrophoresis and labeled by nick translation (5×10^7 cpm/μg). The probe was denatured and allowed to renature to a Cot of 10^{-4}, and the remaining single-stranded sequences were purified through hydroxyapatite chromatography. DNA thus purified was able to self-anneal to approximately 50% as assayed by S1 nuclease digestion. 0.5 ng of this DNA was annealed to 6 mg salmon (■), mouse (○) or hamster (△) sonicated DNA as described in Experimental Procedures. For the lower Cot values of salmon DNA (●), 0.05 ng of the probe was annealed with different amounts of DNA in 0.1 ml reaction volumes for 1 hr and processed as before.

methods of DNA transformation may be used. Whatever the method, the integration events are likely to be quite complex.

Experimental Procedures

Cells and Plasmids

Mouse Ltk$^-$ aprt$^-$ cells and 143, a tk$^-$ human osteosarcoma cell line, provided by C. Croce, were maintained in Dulbecco's modified Eagle's medium containing antibiotics (DMEM) and 10% calf serum. Chinese hamster A29 cells containing a mutant dhfr resistant to mtx (Flintoff et al., 1976) were obtained from L. Siminovitch, and were propagated in DMEM supplemented with 3 times the usual concentration of nonessential amino acids, 10% calf serum and 20 μg/ml of amethopterin (mtx). The derivation of ϕX-4 was as previously described (Wigler et al., 1979b).

Plasmids poT and ptk2 (Hanahan et al., 1980) and pchtk2 (Perucho et al., 1980) are pBR322 derivatives constructed as previously described. T18g, a pBR322 derivative containing the yeast Leu 2 gene and a tRNA suppressor, was a gift from J. Broach (SUNY, Stony Brook).

Transformation, Co-transformation, Amplification and Selection

Transformation of Ltk$^-$ aprt$^-$ for tk$^+$ phenotype was performed with the purified HSV-1 tk gene contained in the recombinant plasmid ptk2 (Hanahan et al., 1980) using the calcium phosphate method of Graham and ver der Eb (1973) as previously described (Wigler et al., 1979a). Co-transformation of Ltk$^-$ aprt$^-$ cells with unselected marker sequences was achieved by adding 1 μg of marker to 20 μg of carrier DNA containing 1–10 ng of the tk gene (Wigler et al., 1979b). 143 cells were transformed with 100 ng of Sal I-cleaved ptk2 plasmid and co-transformed with 1 μg of purified Eco RI-A fragment of adenovirus 2 DNA mixed with 20 μg of carrier DNA per dish. Selection in HAT medium was as described (Wigler et al., 1977).

Transformation of Ltk$^-$ aprt$^-$ cells for resistance to mtx was performed with 20 μg of genomic DNA from the hamster cell line A29. For co-transformation, 1 μg of circular T18g plasmid was added per 20 μg of A29 DNA. Selection of clones resistant to increasing concentrations of mtx was as described (Wigler et al., 1980).

Selection of tk$^-$ revertants from tk$^+$ transformants was achieved by seeding 10^2, 10^3, or 10^4 cells per dish in DMEM containing 10% calf serum and 30 μg per ml of BUdR. Individual resistant colonies were isolated by the use of cloning cylinders and grown in mass culture. After about 30–40 generations, BUdR-resistant clones were plated back in HAT medium, and only those clones which did not form colonies (fewer than 10^{-6}) were used for further studies. In each case that tk$^-$ revertants were generated, we also generated tk$^+$ subclones in HAT from the same parents for use in blot analysis.

Transformation of E. coli χ1776 for ampicillin resistance was accomplished using a high efficiency protocol (D. Hanahan, manuscript in preparation).

Extraction, Restriction Digestion and Ligation of DNA

High molecular weight DNA from cultured cells was prepared as previously described (Wigler et al., 1979a). DNA preparations to be

used for animal cell and E. coli transformations were further digested for 2 hr at 37°C with 20 μg/ml RNAase A and 1 U/ml RNAase T1 in 10 mM Tris-HCl and 10 mM EDTA (pH 7.6). After RNAase treatment, NaCl and SDS were added to 0.2 M and 0.2% respectively, and the DNAs were again treated with proteinase K (100 μg per ml) at 37°C overnight. DNAs were extracted twice with aqueous buffer-saturated phenol:chloroform:isoamyl alcohol, 25:24:1 (v/v), and once with chloroform:isoamyl alcohol, 24:1. High molecular weight DNA was isolated by mixing the aqueous phase with 2 vol of ethanol and removing the fibrous DNA precipitate with sterile glass rods, washed sequentially with 70 and 100% ethanol, and dissolved in sterile 1 mM Tris-HCl and 1 mM EDTA (pH 7.6). Plasmid DNAs were isolated from cultures of E. coli χ1776 as previously described (Hanahan et al., 1980).

Cellular or plasmid DNAs were digested with various restriction endonucleases under conditions recommended by the supplier (New England Biolabs or Bethesda Research Laboratories). Digestions were performed at an enzyme to DNA ratio of 2–3 U/μg for 2–4 hr at 37°C. Where necessary, DNAs were extracted once with chloroform: isoamyl alcohol, ethanol-precipitated and dissolved in the appropriate buffers. Cyclization of restriction endonuclease-cleaved cellular DNA was performed at 4°C for 12–24 hr with T4 DNA ligase (Bethesda Research Laboratories) at a DNA concentration of 5 μg/ml with 2 units of enzyme per ml in buffer recommended by supplier.

Filter Hybridization

Restriction endonuclease-cleaved DNAs were electrophoresed in horizontal agarose slab gels in 40 mM Tris, 4 mM sodium acetate, 1 mM EDTA (pH 7.9) (electrophoresis buffer). DNA fragments were transferred to nitrocellulose sheets, hybridized and washed as previously described (Wigler et al., 1979b).

DNAs used as probes were labeled with ^{32}P by nick translation (Maniatis, Jeffrey and Kleid, 1975) using DNA polymerase I (Worthington) as described (Pellicer et al., 1978). When restriction endonuclease DNA fragments were used as probes, they were purified by electrophoresis in agarose gels. Gel strips containing the fragment were placed in dialysis bags filled with electrophoresis buffer and electroeluted for 4–6 hr at 100–150 V. The DNA solution was applied to a DEAE-cellulose column and washed with electrophoresis buffer containing 0.1 M NaCl, and the DNA was eluted with the same buffer containing 1 M NaCl. The fractions containing DNA were pooled, diluted 1:1 with water and ethanol-precipitated. The final precipitate was dissolved in 1 mM Tris-HCl, 1 mM EDTA (pH 7.6).

Solution Hybridization

Gel-purified DNA fragments from rescued plasmids were labeled by nick translation, and "foldback" DNA was removed by hydroxyapatite chromatography (Pellicer et al., 1978). Denatured DNA probes were annealed with denatured, sonicated DNA from various species (average size 200–600 bp) in 0.4 ml of 1 mM Tris-HCl (pH 7.6), 1 mM EDTA and 0.4 M NaCl at 68°C under parafilm oil. Aliquots (40 μl) were removed at different times and diluted in 1 ml of 30 mM NaOAc (pH 4.5), 0.1 M NaCl, 1 mM $ZnCl_2$ and 50 μg/ml of bovine serum albumin. The samples were assayed for duplex formation by digestion with S1 nuclease as described (Pellicer et al., 1978).

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