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Analysis of Stable and Unstable Viral Forms in SV40-Infected Human Keratinocytes

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We analyzed the state of the genomic DNA of the papovavirus SV40 in human keratinocytes as viral-infected cells gradually acquired a transformed phenotype over time. Initially, the vast majority of the viral DNA is maintained either in a full-length supercoiled form or as truncated subgenomic fragments with little evidence of integration. However, analyses of clonal populations revealed great heterogeneity and instability of the viral DNA, and we were able to isolate one clonal subpopulation in which integrated forms of the virus appeared to predominate. Similarly, uncloned populations eventually ceased production of the "free" viral DNA after several years in culture and instead came to display tandemly repeated SV40 copies at a single host integration site. Interestingly, Bgl II digestion of host DNA generated restriction fragments containing the integrated SV40 DNA, which were of differing sizes in cultures at the 144th vs the 163rd serial passage suggesting modification or rearrangement of sequences at or near the integration site. Host sequences flanking the integrated viral DNA at the 163rd serial passage have been isolated on restriction fragments generated by Eco RI, Bam HI, and Hpa II digestion. These analyses suggest that the integrated virus is linearized near the Bgl I site and contains a large deletion in the SV40 early region at one of the viral-host junctions.

Key words: SV40-transformed keratinocytes, state of SV40 DNA, clonal variability, structure of integrated SV40, viral dependence of transformation

The role of oncogenic viruses as research tools for studying transformation both in vivo and in vitro has greatly expanded in recent years as more has become known about their biochemistry. Over the last several years, our laboratory has described the transformation of human epidermal keratinocytes by the oncogenic virus SV40 [1,2]. A number of previous studies have examined the state of integration of SV40 DNA primarily in fibroblasts of rodent and, to a lesser extent, human origin, but few, if any, investigations have been carried out on terminally differentiating human cells (see [3,4] for a review). The purpose of the present study is twofold: (1) to document

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SV40 integration in the keratinocyte host system and (2) to discern time-related changes in quantitative or qualitative aspects of the pattern of SV40 sequences that may be relevant to the evolution of the transformation process. Our results indicate that the infected keratinocytes exhibit an extended period of instability with respect to both normal and defective SV40 replication, but that eventually the viral DNA stabilizes as a single integrated form. The work presented here is also intended to form the basis for future studies in which modulation of the epidermal phenotype might be examined through experimental manipulation of viral integration.

MATERIALS AND METHODS

Cell Lines

Infection of human epidermal keratinocytes by SV40 virus and the growth properties of the resulting cell lines have been described in detail elsewhere [2]. In the present study, line 98 cells, which have been in continuous culture for more than 5 yr, were used. The clonal sublines were all derived at the 23rd serial passage (~ 1½ yr) by selection in soft agar. Sublines 33 and 34 were derived by a second round of selection from a common clone (03); in the same way, 62 and 64 were both derived from clone 06, and 72 was derived from clone 07. The colony forming efficiencies for some of these clonal populations has been described [1].

Blot Hybridization

DNA isolated from the various cell lines was run on 1% agarose gels, transferred to nitrocellulose sheets, and hybridized to a nick-translated ^{32}P-labeled SV40 probe by standard procedures as described [5]. Ten micrograms of cellular DNA was run in each gel lane except in Figure 4, where 20 μg per lane was run because of the low signal produced by the putative host-virus junctional sequences. Hybridization was carried out under stringent conditions using 0.1 × SSC at 50°C to wash the nitrocellulose.

RESULTS

The structural arrangement of SV40 DNA in clonal populations of viral-infected keratinocytes was analyzed by DNA blot hybridization. Figure 1 shows the SV40-DNA-containing fragments generated by digestion of cellular DNA with Bgl II, an enzyme with no SV40 restriction sites. These results show that the pattern of restriction fragments and the viral DNA content varied widely among the different clones, even within a clonal lineage (eg, Ag06 vs Ag62 or Ag07 vs Ag72). In all the cell lines tested a large proportion of the viral DNA was present in species that comigrated with full-length circular viral DNA; digests with Eco RI (one SV40 restriction site) did not contain these species, but instead exhibited a single band comigrating with linear viral DNA as the major form (Fig. 2). The Bgl II digests in Figure 1 also show many minor bands of both sub- and supragenomic size. Digestion with Eco RI reduced the number of supragenomic species and produced many more subgenomic bands, accentuating the differences between the clones. However, some of the clones (eg, Ag33, Ag34, and Ag64; Fig. 2) exhibited prominent bands larger than linear SV40 DNA even after Eco RI digestion, suggesting that these may represent integrated forms.

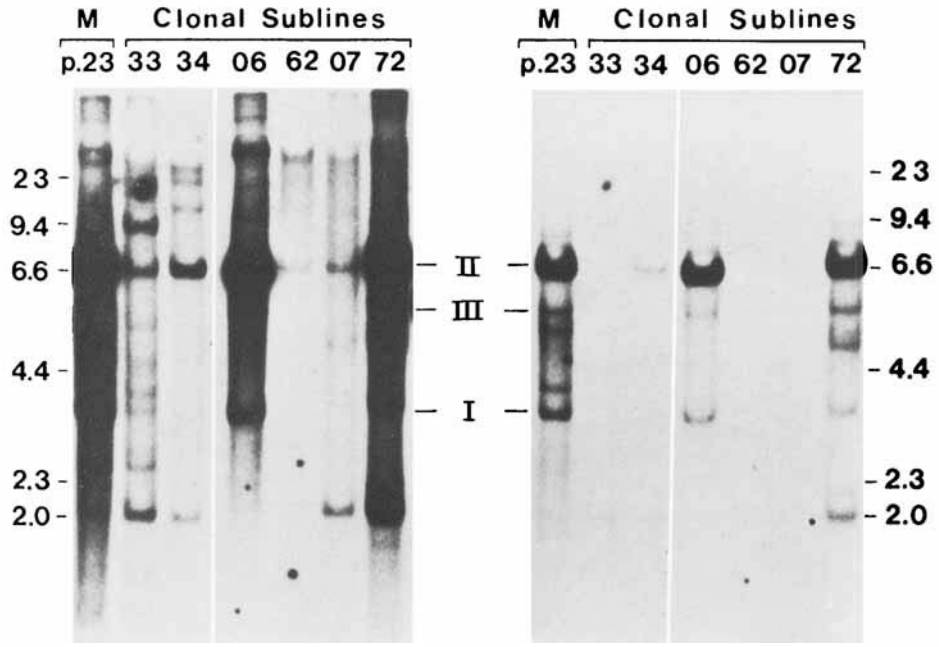

Fig. 1. SV40 sequences in DNAs from anchorage-independent clonal sublines of SV40-infected keratinocytes after Bgl II digestion. Thirty-three and 34 are double selectants derived from a common clonal parent, 03 (not shown). Sixty-two and 72 are double selectants derived from the clonal parents 06 and 07, respectively. The unselected parental population is also shown (M). Exposures of the film for 24 and 72 hr are shown in the right- and left-hand panels, respectively. The migration of Hind III-digested λ markers (in kilobases) is indicated at the outer edge of each panel; the migration of supercoiled (form II), linear (form III), and nicked circular (form I) full-length SV40 DNA is shown between panels.

To further elucidate features of the viral DNA that might be of interest with respect to transformation, we analyzed the DNA from one clone (Ag34) in more detail because of its apparently low content of free viral DNA (Figs. 1, 2). Digestion of Ag34 DNA with three different restriction enzymes that do not cut SV40 DNA produced 5–6 major bands ranging in size from 2.1 to about 30 kilobases (Kb) (Fig. 3). Of these, only the 2.1-Kb fragment was present in all three digests, whereas the sizes of each of the other SV40-containing fragments varied from one digest to the next, indicating that these species may contain DNA of host origin. A 2.1-Kb fragment was also present in Bgl I digests, an enzyme with one SV40 restriction site (Fig. 3A). This enzyme produced one fragment comigrating with full-length linear SV40 DNA as would be expected if one or more of the supragenomic fragments contained tandem copies of the SV40 genome. Figure 3B shows that similar fragments were also produced in each of the five digests using different single-cut enzymes, but that no such fragment existed after digestion with an enzyme with two restriction sites (Pst I). For enzymes that do not cleave SV40, several were found to generate 2.1-Kb fragments (ie, Bgl II, Bst EI, Sst I; Fig. 3), although at least one other such enzyme (Ava I) does not (not shown). Fragments of this size were also generated by enzymes with single cleavage sites near the SV40 origin of replication (Bgl I, Kpn I, Hpa II),

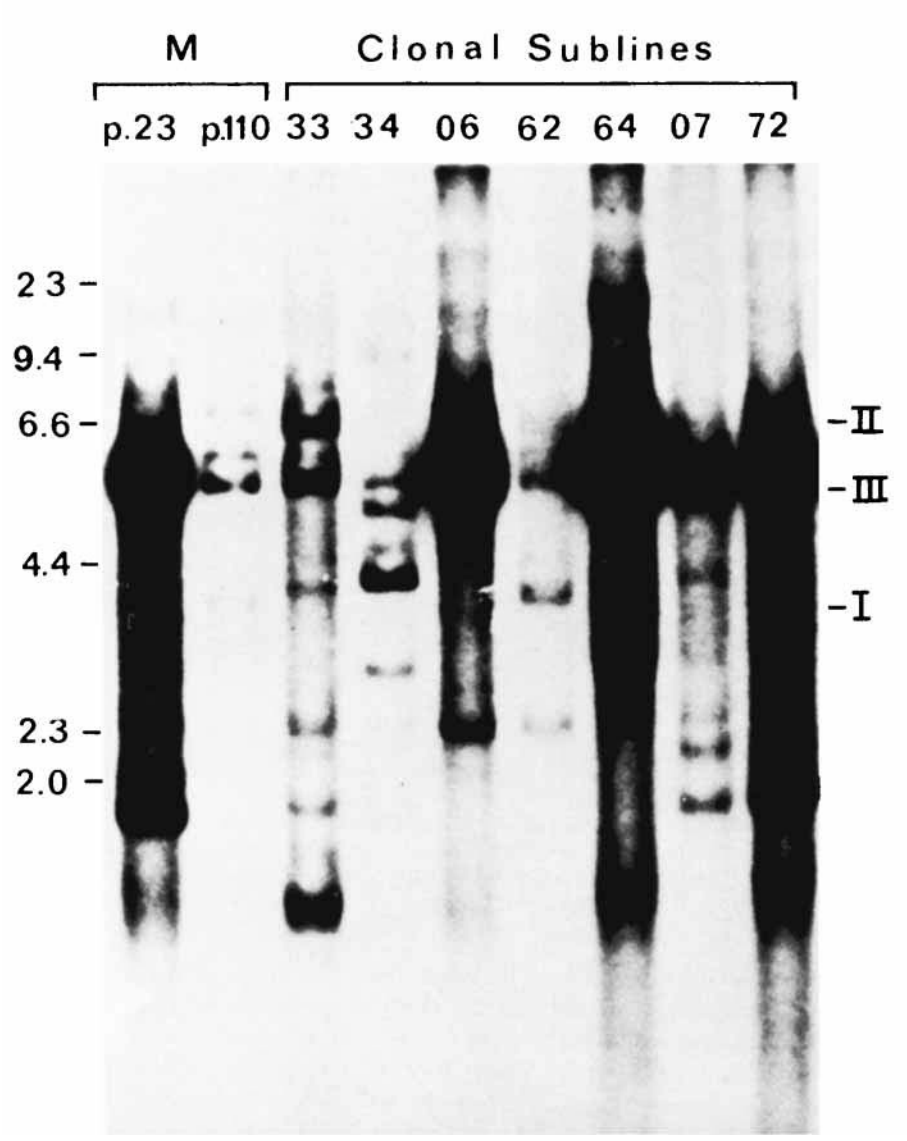

Fig. 2. SV40 sequences in DNAs from anchorage-independent clonal sublines of SV40-infected keratinocytes after Eco RI digestion. The cell subline designations and markers are the same as in Figure 1, but the parental population (M) is shown at two passage levels.

but not by those with sites distal from the origin of replication (Eco RI, Bam HI). Figure 3C shows that no SV40-containing species that were electrophoretically separable from high molecular DNA (ie, episomal forms) could be detected in undigested Ag34 DNA.

Since the viral DNA in the infected keratinocytes became greatly simplified after long-term culture (Fig. 1), high-passage cultures were examined to determine the structure of the residual viral DNA. Figure 4A shows Bgl II digests of DNA from cultures at the 144th and 163rd serial passages. Passage 144 cells showed two bands

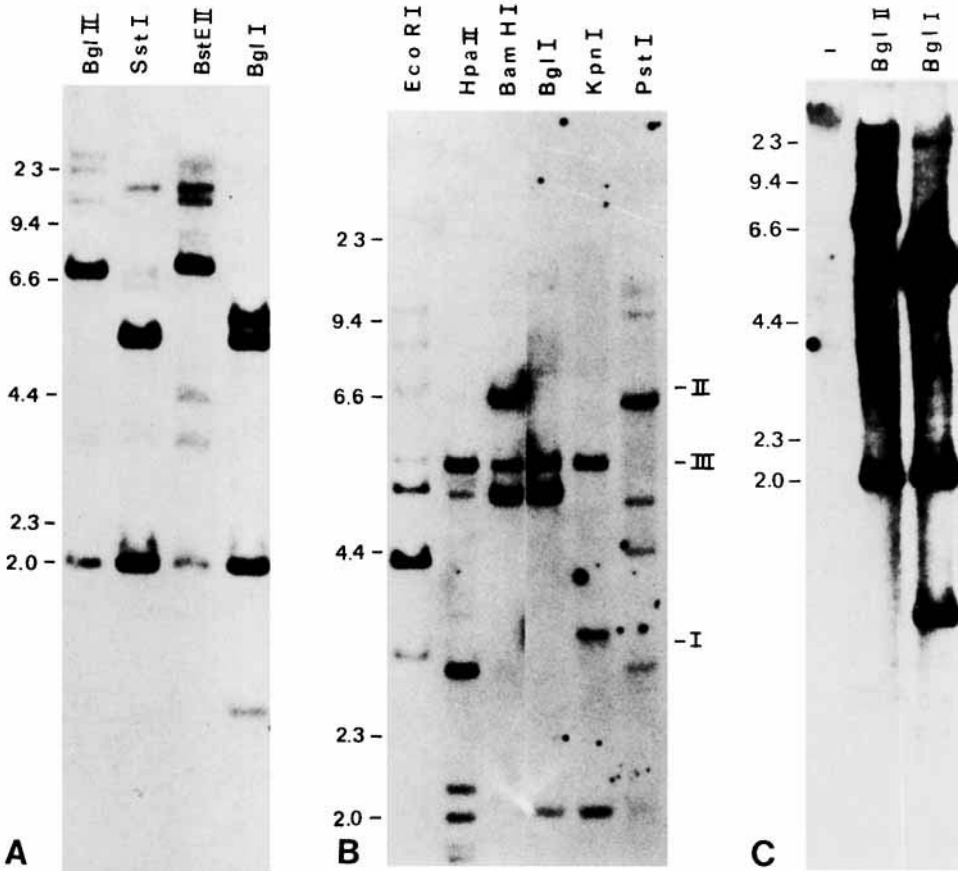

Fig. 3. SV40 sequences in digests of DNA from clonal subline 34 using a series of restriction enzymes with either no SV40 restriction sites (A) or with one SV40 restriction site (B); a Bgl I digest is shown at the right of panel (A) for comparison. Panel (C) shows undigested Ag34 DNA; Bgl-I- and Bgl-II-digested DNAs were run concurrently for comparison. The autoradiogram in (C) is overexposed to visualize minor bands. Numbers at the left of each panel indicate the migration of λ markers (in kilobases).

of about 30 and 6.8 Kb in length, but after an additional 19 passages, the same cultures showed only a single band of about 23 Kb. Analysis of the higher passage cultures using four different single-cut enzymes showed a band equivalent to full-length linear viral DNA in each case as well as SV40-containing species of variable size depending upon the enzyme used (Fig. 4B). Digestion with Bgl II and Bst EII, two no-cut enzymes for SV40, produced single but different high-molecular-weight bands (28 and 23 Kb, respectively). Double digestion with combinations of one-cut enzymes produced bands of sizes expected for restriction of full-length SV40 DNA (~4.5 Kb for Eco RI + Bam HI and ~3.8 Kb for Eco RI + Hpa II).

To test the dependence of transformation on the continued presence of the integrated virus, a number of clonal populations were derived from the high-passage (p. 170) cells by selection of populations derived initially from microtiter wells into

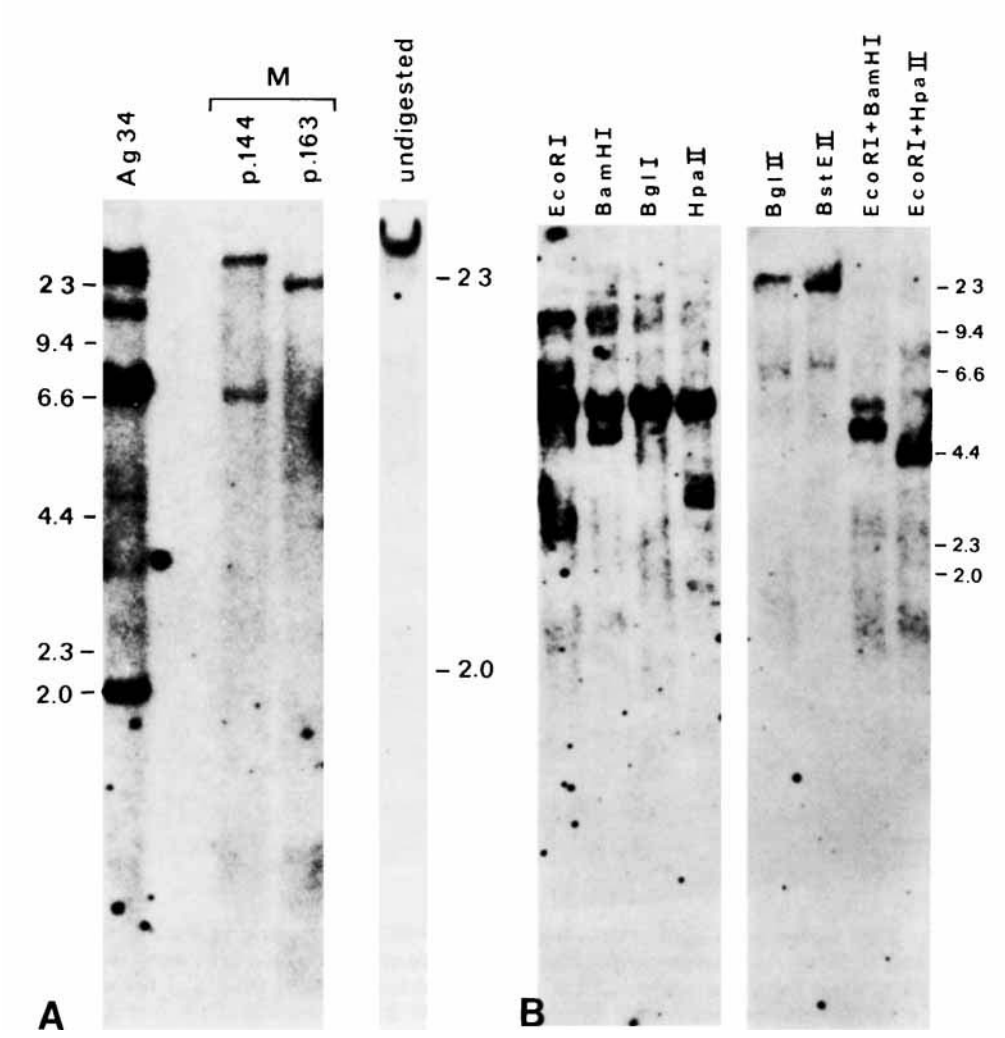

Fig. 4. SV40 sequences in the unselected population of infected keratinocytes at high passage. Panel (A) shows DNA from cells at the 144th and 163rd passage levels after Bgl II digestion; Ag34 cleaved with Bgl II is shown on the left for comparison. Offset lane at right shows undigested DNA from the unselected population (p. 160). The migration of λ markers is shown at the right of each panel. Panel (B) shows DNA from cells at the 163rd passage digested with a series of enzymes that cleave SV40 DNA at one site (Eco RI, Bam HI, Bgl I, Hpa II) or have no SV40 restriction sites (Bgl II, Bst E II).

which 1–3 cells had been seeded. Each population was then reselected in the same way and then scored for the percentage of SV40-T-antigen-positive cells by immunofluorescence. Ten to fifteen subpopulations at each of 15 selection rounds were examined in this way, and then ten of the final selectants were analyzed by blot hybridization using Bgl II to digest the DNAs. These experiments showed that (1) while there was considerable fluctuation in the percentage of T-antigen-positive cells from one selectant to the next, a stable population of T-antigen-negative cells was never obtained and (2) that the integrated virus was stable in the selectants.

DISCUSSION

In this report we have conducted a detailed analysis of changes in the state of SV40 DNA as the transformed phenotype evolves in viral-infected human keratinocytes. These studies showed that most of the viral DNA in the infected cells was initially present as free full-length circular forms, but there are also both sub- and supragenomic DNAs with some of the higher-molecular-weight forms probably representing integrated SV40 sequences. Analyses of clonal populations revealed differences in both the quantity and pattern of SV40-DNA-containing restriction fragments in different clones. Furthermore, although each cloned line maintained its characteristic viral DNA pattern over at least ten subcultures (for example, the Bgl II digests of Ag34 DNA in Figs. 1, 3A, and 4A represent a span of six subcultures), clonal sublines derived by a second round of agar selection exhibited the same quantitative and qualitative heterogeneity as did the primary clones indicating that the observed variability was actively generated rather than wholely a process of passive selection. However, a certain general pattern was consistently observed in that the amount of viral DNA gradually diminished over time postinfection with only integrated forms being preserved after long-term culture; only a single integration site was evident after 4½ yr in culture. Our analysis of the integrated viral DNA in Figure 4 indicated a tandemly repeated full-length SV40 genome in a head-to-tail configuration, an arrangement that would be expected to produce linear (form III) viral DNA upon digestion with one-cut enzymes; the much weaker bands of variable size are presumed to represent the viral-host junctional sequences. Further confirmation of this arrangement can be found in the double digests in Figure 4B, where only the large fragments expected from a head-to-tail arrangement were seen in the Eco RI/Bam HI and Eco RI/Hpa II digests; head-to-head or tail-to-tail arrangements would have generated fragments of different sizes depending upon the location of the repeat on the genome. As estimated from the size of the smallest fragment produced after digestion with a no-cut enzyme (ie, about 23 Kb for the Bst EII digest in Fig. 4B), the integrated virus could represent at most four full-length virus copies. It is also of interest that the Bgl-II-generated restriction fragments bearing the integrated viral DNA are apparently different at the two passages shown in Figure 4. At least two possibilities are suggested by these findings: (1) excision of some of the integrated viral DNA resulting in a Bgl II fragment of smaller size in cells at the 163rd passage or (2) modification or rearrangement of host sequences at or near the integration site over a period of several months in culture. The possible nature and extent of these changes is unknown.

The 2.1-Kb fragments in the digests of Ag34 DNA (Figs. 1–3) apparently arise from viral sequences integrated into host DNA because they are not observed apart from undigested high-molecular-weight DNA, and they are seen only in some digests where enzymes that do not cleave SV40 DNA were used; the latter observation is also true for Ag72 (compare the Bgl II digest in Fig. 1 with the Ag72 Sma I digest in ref [1]). It may be that a number of enzymes can generate fragments close to 2 Kb in size. This would explain not only the ubiquity of these fragments but also differences in their relative abundance in different digests (Fig. 3).

Figure 5 illustrates a simple scheme based on head-to-tail tandem replication of full-length SV40 as has generally been found for integrated forms of SV40 or polyoma [3]. With minor modification, this scheme can account for the restriction patterns observed in Figure 4B, for example, the two Eco RI fragments and the small sizes of

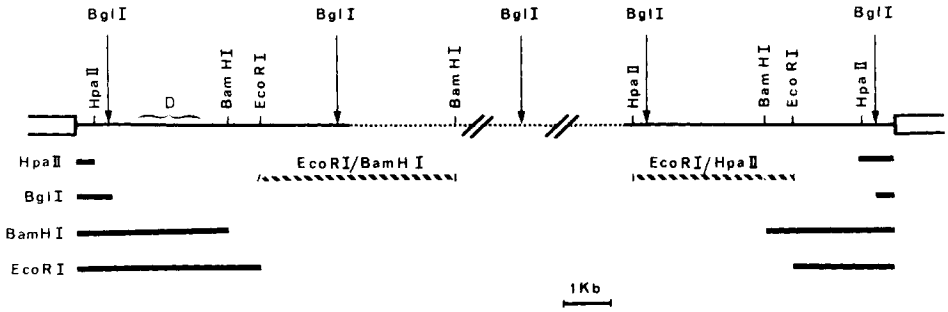

Fig. 5. Schematic representation of the integrated SV40 in high-passage cells based on the restriction fragments in Figure 4. The model shows four tandemly repeated full-length copies of SV40 DNA in a head-to-tail configuration with integration near the viral Bgl I site; host sequences are represented by the open bars on either side. Approximate sizes of the viral portions of the various restriction fragments containing viral-host junction sequences are shown by the bars below the schematic. The stippled bars represent the respective Eco RI/BamHI and Eco RI/HpaII fragments generated from the linearized viral genomes. The bracketed region, D, represents the probable location of deleted viral material.

the viral segments in the Hpa II digests. However, from the model in Figure 5, it is surprising that the Bam HI digest produced only one distinct junctional band since two fragments, each containing large amounts ($\sim$2.7 Kb) of viral DNA, should have been generated. Also, the Eco RI/Bam HI digests that show two bands should have produced at least three bands with enough viral DNA to be detected: one band of 4.5 Kb containing only viral DNA and two junctional bands containing about 2.7 and 1.7 Kb of viral material based on junction at the Bgl I site. Similarly, at least one of the fragments generated by Hpa II is too small to be derived from intact virus linearized at the Bgl I site. The simplest model that would accommodate these findings would have to include a fairly large deletion of at least 2 Kb in the early region at one end of the integrated virus.

Our inability to derive clonal sublines of T-antigen-negative cells or sublines in which the viral DNA content is further reduced indicates that maintenance of the transformation process is under the control of this single integration. It should be noted that in these experiments, a passive selection technique was used, which, unlike the anchorage-independent conditions used to derive the clonal populations in Figures 1–4, imposed no obvious selective pressures on the cell populations examined. The possibility exists that environmental pressures may actively cause amplification and rearrangement of the viral sequences as has been suggested for SV40-transformed mouse cell lines [5], and the apparent stability of the viral genome at high passage may therefore be related, at least in part, to the selection process.

The presence of large quantities of viral DNA during the early passages may promote the transformation process. In another report, where human keratinocytes were transfected with a cloned restriction fragment containing the SV40 early region, the resulting transformants were immortalized but did not express other properties seen when whole virus is used, eg, growth independence from feeder layers or anchorage to solid substrates [6]. These differences are probably attributable to the fact that such transfected DNAs are present in low copy number. In this regard, we have previously shown that the clonal populations showing the highest content of

viral DNA also show the highest colony forming efficiency in soft agar [1]. Recently, Blanck et al [7] reported that in mouse cell lines the appearance of defective SV40 DNA correlated with selection of sublines for anchorage independence.

The persistence of such large amounts of free viral DNA over long periods of time also distinguishes this sytem from the more commonly studied rodent cells, where there is generally a much more rapid loss of unintegrated SV40 DNA [5].

Other biologic characteristics of the infected keratinocytes are temporally correlated with the progression to a state where the viral DNA is present in a more simplified and stable configuration. For example, keratin markers characteristic of stratified epithelium become increasingly less inducible by 5-azacytidine [8], while, conversely, synthesis of fibronectin becomes greatly enhanced [9] with serial passage. The dependence of expression of these and other properties upon integration might now be tested by the use of agents that prevent integration such as interferon [10] or xanthate compounds [11].

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