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Nucleosomal packaging of the thymidine kinase gene of herpes simplex virus transferred into mouse cells: An actively expressed single-copy gene

(DNA transfer/gene transfer/nucleosomes/active genes/Southern blot method)

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ABSTRACT **We have studied the nucleoprotein structure of the herpes thymidine kinase gene introduced into mouse Ltk$^-$ aprt$^-$ cells by means of DNA-mediated gene transfer. Using the technique of Southern blotting, we examined staphylococcal digests of the nuclei from three relatively stable transformants that contain one or less integrated copies of the thymidine kinase gene per haploid genome. Our experiments show that, under selection for the active expression of this gene, it is packaged in nucleosomes with a repeat length identical to the average for the host mouse sequences.**

The herpes simplex viruses (HSVs) code for a thymidine kinase (TK) and this genetic information has been transferred to TK-deficient cells (tk$^-$) by infection with temperature-sensitive mutants of these viruses (1), with UV-inactivated HSV-1 or HSV-2 (2), and, more recently, with fragments of the DNA from these viruses (3–5). The phenomenon of gene transfer into eukaryotic cells by pure DNA (transformation) was soon extended to several cellular eukaryotic genes (6–9).

Although information on the integration and expression of the transferred DNA is emerging (10–13), to our knowledge, a most basic question has not yet been asked: What is the nature of the nucleoprotein structure containing the new genetic information? In this paper, we report that, when transferred into mouse cells, the HSV thymidine kinase gene (*tk*) is protected from staphylococcal nuclease attack in the same fashion as the host DNA packaged in nucleosomes. We infer that this actively transcribed foreign gene is packaged in the same manner.

Because we were interested in learning not only whether the transferred gene is packaged in nucleosomes but also whether the gene packaged in this manner is being transcribed, we will show in the following sections that: (*i*) the viral TK activity accounts for the survival of these cells in selective medium; (*ii*) the three lines do not contain more than one copy of *tk* per haploid genome; and (*iii*) these cells show a relatively stable TK$^+$ phenotype.

MATERIALS AND METHODS

Cell Culture. Mouse Ltk$^-$ aprt$^-$ cells are derivatives of the LM(tk$^-$) (14) and were obtained from S. Silverstein and R. Axel. Mouse LM(tk$^+$) cells were obtained from the American Type Culture Collection (Rockville, MD). All of these cells were maintained in antibiotic-free Eagle's minimal essential medium with twice the standard concentration of amino acids and vitamins and 10% heat-inactivated fetal calf serum (hereafter designated "2× medium"). Other media were: HAT medium [hypoxanthine (15 μg/ml)/aminopterin (1 μg/ml)/thymidine (5 μg/ml)/glycine (15 μg/ml) in 2× medium (15)]; HT medium, identical to HAT medium but lacking aminopterin; and BU medium, 2× medium supplemented with 30 μg of BrdUrd (Sigma) per ml. Cell lines were grown as monolayer cultures in Corning plastic flasks and dishes at 36°C under a humidified atmosphere of 5% CO_2/95% air.

Isolation of Transformant Cell Lines. The cells from a given colony were removed from the dish by incubating the colony with 5-mm discs of Whatman no. 50 paper that had been soaked in 0.1% trypsin and 0.01% EDTA in Dulbecco's phosphate-buffered saline without calcium or magnesium (buffer A); the filters were transferred to a 60-mm dish containing HAT medium. After 8–12 days the colonies growing in these dishes were trypsinized, and the cells were then plated at several dilutions in 100-mm dishes containing HAT medium. A single colony from an appropriately sparse dish was then removed as described above and transferred to a 60-mm dish. The process was repeated again.

Transformations. Transformations were carried out by a modification (6) of the $CaCl_2$ method first described by Graham and van der Eb (16).

HSV-1 *tk* DNA. Whole HSV-1 DNA was the gift of Peter Howley. The *Escherichia coli* strain LE 392 containing the 3.5-kilobase (kb) *Bam*HI restriction fragment, bearing the HSV-1 (clone 101) *tk* gene, ligated into the *Bam*HI site of pBR322 (17) was the gift of Lynn Enquist and George Vande Woude. The recombinant plasmid was purified by the acid/phenol extraction method (18). All experiments involving the propagation of recombinant DNA molecules in bacterial and animal cells were performed in compliance with National Institutes of Health guidelines for recombinant DNA research.

TK Activity of Extracts from Cells. Cells grown in monolayer in HAT medium were trypsinized into buffer A, washed twice in buffer A, and then lysed in 300 μl of 0.15 M KCl/10 mM Tris·HCl, pH 7.4/1 mM $MgCl_2$/1 mM 2-mercaptoethanol/50 μM thymidine/0.5% Nonidet P-40 at 4°C. The mixture was centrifuged at 20,000 × *g* for 20 min at 4°C. The supernatants were assayed by the procedure of Lee and Cheng (19) except that the substrate was 50 μM [^{3}H]thymidine. In parallel reactions, 100 μM BrdCyd was added and the inhibition of the conversion of [^{3}H]thymidine to [^{3}H]thymidylic acid was measured (20).

Preparation of Cellular DNA. DNA from transformed cell lines was obtained from trypsinized cells that had been grown in monolayers in flasks. The cells (1–2 × 10^8) were washed twice with buffer A and resuspended in 0.5–1.0 ml of buffer A. DNA was then prepared as described (10).

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Abbreviations: HSV, herpes simplex virus; TK, thymidine kinase; *tk*, thymidine kinase gene; buffer A, 0.14 M NaCl/3 mM KCl/8 mM sodium phosphate, pH 7.2; SSC, 0.15 M NaCl, 0.015 M sodium citrate (pH 7.2); kb and kbp, kilobase(s) and kilobase pair(s).

Preparation and Electrophoresis of Nucleosomal DNA. Nuclei from Ltk$^-$ aprt$^-$ cells (growth in 2× medium) and transformants (grown in HAT medium) were obtained from cells grown in mass culture in monolayer. The cells were trypsinized and washed twice in buffer A. The washed cells were resuspended in 0.25 M sucrose/10 mM Tris·HCl, pH 8.0/1 mM $MgCl_2$ and were then lysed by the addition of 0.2% Nonidet P-40 (Bethesda Research Laboratories, Rockville, MD) at 4°C. This mixture was centrifuged at 8000 × *g* for 10 min at 4°C. After the second Nonidet P-40 wash in the same buffer the nuclei were resuspended in the same buffer without Nonidet P-40 and spun again. The nuclei were then washed in 0.25 M sucrose/10 mM Tris·HCl, pH 8.0/0.1 mM $CaCl_2$, centrifuged, and resuspended in this last buffer at an A_{260} of approximately 10–20 per ml. Within 15 min, the nuclei were digested at 37°C with staphylococcal nuclease (≈10 μg/ml; 25,000 units per mg, Worthington). The reaction was stopped by chilling in ice and the addition of a 200-fold excess of Na_2EDTA (pH 8.0) and 0.5% $NaDodSO_4$. Extent of digestion was monitored as described (21). The suspension was then brought to 0.5 M in NaCl, proteinase K was added to a final concentration of 100 μg/ml, and the mixture was incubated at 37°C for 2 hr. The nucleosomal DNA was extracted once with 1.5 vol of phenol/chloroform/isoamyl alcohol, 50:24:1 (vol/vol), and once with 1 vol of chloroform/isoamyl alcohol, 24:1 (vol/vol). The DNA was concentrated to 2 mg/ml by ethanol precipitation and centrifugation. Approximately 60 μg of DNA in quarter strength Tris acetate/EDTA buffer (22) containing 5% Ficoll 400 (Pharmacia) and 0.01% bromophenol blue was loaded on a 6-mm-thick vertical 1.2% agarose slab (22 × 17 cm) gel in the Tris acetate/EDTA system. Electrophoresis was at 20°C for 5 hr at 2.5 V/cm.

Electrophoretic Analysis of Restriction Nuclease Digests. Restriction endonucleases used were commercial preparations from New England BioLabs. Agarose gel electrophoresis was as described (23).

Filter Hybridizations. The transfer of DNA from the agarose gels to nitrocellulose filters (24) and the filter hybridizations were described (23). Two probes were used: (*i*) whole plasmid bearing both the pBR322 and HSV-1 *tk* sequences (pBR322-tk) and (*ii*) the 3.5-kb *Bam*HI *tk* fragment. The latter was prepared from the former by digesting pBR322-tk with *Bam*HI, running a preparative agarose gel, cutting out the band bearing the *tk* fragment, and removing the DNA from the agarose by a modification of the method of Vogelstein and Gillespie (25). The major modification was elution of the DNA from the glass beads with 0.2 M NaCl/0.02 M Tris·HCl, pH 9/1 mM Na_2EDTA at 68°C for at least 5 min.

^{32}P-Labeled probes were prepared by nick-translation (26) as described (24). Specific radioactivity of the probes ranged from 0.5 to 1.2 × 10^8 cpm/μg of DNA.

RESULTS

Transformation with the HSV-1 *tk* Genes. Ltk$^-$ aprt$^-$ cells were transformed with the HSV-1 *tk* gene wholly contained in a 3.5-kb *Bam*HI fragment [*tk* fragment (3)]. In a typical transformation experiment, only a fraction of 1 μg of the *tk* fragment was added to 40 μg of salmon sperm DNA. The transformation frequency per equivalent of *Bam*HI fragment was 1 per 100 pg for the total *Bam*HI digest of HSV-1 DNA and 1 per 20–40 pg in the case of uncleaved pBR322-tk plasmid, *Bam*HI cleaved plasmid, and purified 3.5-kb *Bam*HI fragment. No colonies were obtained with Ltk$^-$ aprt$^-$ DNA, salmon sperm DNA alone, or 1 or 5 μg of pBR322 in addition to the salmon sperm DNA.

For the purposes of this study we selected three cell lines hereafter designated "1-2-9," "1-2-12," and "1-2-15." The first two were obtained by transformation with *Bam*HI-cleaved HSV-1 DNA; the last transformant was obtained by transforming the Ltk$^-$ aprt$^-$ cells with uncleaved pBR322-tk.

The TK Activity Is of Viral Origin. Cooper (27) has shown that BrdCyd is a good substrate for HSV TK and a poor substrate for cellular kinases. We used a standard assay to show that BrdCyd can compete effectively with the phosphorylation of thymidine in extracts of transformed cells (20).

The TK activities for the four cell lines tested were (in pmol of thymidine converted to thymidylic acid per min per 10^7 cells grown on HAT): 14, 4, 3, and 2 for LM, 1-2-9, 1-2-12, and 1-2-15 cells, respectively. The percentage of enzyme activity found in the presence of 100 μM BrdCyd was (in three separate experiments): 58%, 54%, and 51% for LM cells; 13%, 13%, and 13% for 1-2-9 cells; 11%, 18%, and 20% for 1-2-12 cells; and 19%, 19%, and 18% for 1-2-15 cells. This degree of competition is consistent with that expected for the viral enzyme (3, 20).

Integration of *tk* Sequences. Fig. 1 shows a restriction map for the 3.5-kb *Bam*HI fragment bearing the HSV-1 *tk* gene (17); *Sac* I and *Hin*dIII do not cut this fragment. pBR322-tk is cut only once by *Hin*dIII. The orientation of the *tk* fragment (17) in pBR322-tk is such that, when it is digested with *Eco*RI, the three fragments obtained are 4600, 2300, and 875 base pairs (bp) long.

As evidence for integration, we will show that the restriction pattern of the *tk* fragment in the transformants is different from that shown in Fig. 1, particularly on the ends. We also will show, by the uniqueness of the restriction patterns and by the photographic intensities of the films exposed to the nitrocellulose filter transfers, that only one copy of the *tk* gene per haploid genome is present in 1-2-9, 1-2-12, and 1-2-15. In order to show that the pattern is consistent with only one copy rather than multiple copies, we present the data for several restriction endonuclease digests of each line.

Fig. 2*A* shows the electrophoretic mobilities of the *tk* sequences in the total (nuclear and cytoplasmic) cellular DNA of 1-2-9 (after 100 generations in HAT) digested with *Eco*RI, *Pst* I, and *Bam*HI. Lane 2 shows the expected pattern obtained by cleaving the 3.5-kb *Bam*HI fragment with *Eco*RI (see Fig. 1). With DNA from the transformed cell, cleavage with *Eco*RI yielded two fragments, only one of which, the central 2.3-kb fragment, corresponded to a fragment in lane 2. The simplest explanation for these data is that both flanking sequences have been the sites for recombination events with another DNA. The new 1.4-kb fragment generated is presumably the result of cleavage of the DNA in 1-2-9 at an additional new site now linked by recombination to one of the two original *Eco*RI sites shown in Fig. 1. Our inability to detect an additional fragment corresponding to the other original site would be as expected if the new fragment were either too large (see below and lane 5) or too small (smaller than the smallest fragment in lane 2). In lane 5 a very large fragment, indicated by the arrow, was obtained when 1-2-9 DNA was cleaved with *Bam*HI; the weak hybridization was the result of poor transfer to the nitrocellulose sheet for fragments in this size range.

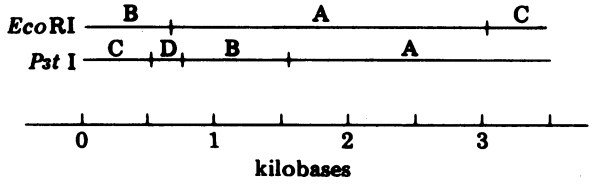

FIG. 1. Map of *Eco*RI and *Pst* I cleavage sites in the 3.5-kb *Bam*HI fragment of HSV-1 (clone 101) DNA (10, 17).

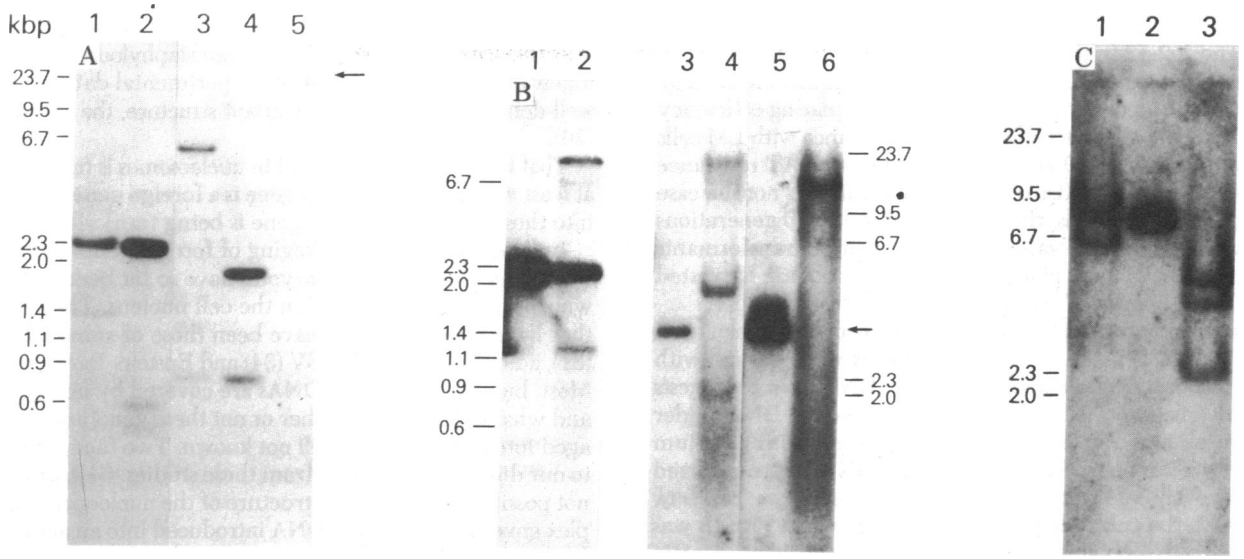

FIG. 2. Electrophoretic pattern of the HSV *tk* sequences in DNA fragments generated by restriction endonuclease cleavage of total cellular DNA from transformants. Restriction endonuclease digests were electrophoresed on 0.7% agarose gels. The DNA was then transferred to nitrocellulose filters and hybridized with ^{32}P-labeled DNAs; autoradiograms are shown. (*A*) ^{32}P-Labeled probe was the *Bam*HI HSV *tk* fragment. Cellular DNAs from transformant 1-2-9 digested with *Eco*RI (lane 1), *Pst* I (lane 3), and *Bam*HI (lane 5) are shown; the arrow points to the weakly hybridizing fragment in lane 5. Digests of 40 pg of the 3.5-kb *tk* fragment with *Eco*RI (lane 2) and *Pst* I (lane 4) are shown as standards. (*B*) ^{32}P-Labeled probe was pBR322-tk. Cellular DNAs from transformant 1-2-12 digested with *Eco*RI (lane 2), *Sac* I (lane 4), and *Hin*dIII (lane 6) are shown. The arrow indicates the position corresponding to 3.5-kb in lanes 3–6. Digests of 60 pg of the 3.5-kb *tk* fragment with *Eco*RI (lane 1), *Sac* I (lane 3), and *Bam*HI (lane 5) are shown as standards. (*C*) ^{32}P-Labeled probe was pBR322-tk. Cellular DNAs from transformant 1-2-15 digested with *Hin*dIII (lane 1) and *Eco*RI (lane 3) are shown. A *Hin*dIII digest of 60 pg of pBR322-tk is shown in lane 2.

Fig. 2 *B* and *C* shows the electrophoretic patterns for the *tk* sequences in digests of the total cellular DNA from cells 1-2-12 (after 200 generations in HAT) and 1-2-15 (after 100 generations in HAT). Furthermore, when 1-2-15 DNA was digested with *Sac* I, a restriction endonuclease that does not cleave pBR322-tk, the pBR322 and *tk* sequences were in a 12-kb fragment, not at the position of uncleaved pBR322-tk (data not shown). In both 1-2-12 and 1-2-15, the introduced sequences have been covalently linked to another DNA (i.e., integrated). The integration patterns shown here for the three transformants have not changed during more than 100 generations in HAT medium and we have not found any differences between the patterns obtained from both nuclear and total (shown in Fig. 2) cellular DNA. Finally, we should point out that, in spite of the integration events, it is not inconceivable that the transferred sequences are still episomal.

The patterns shown in Fig. 2 can also be used to quantitate the number of copies of these sequences per haploid genome. Multiple copies with different integration sites can be ruled out by the uniqueness of these patterns. Multiple copies with identical restriction patterns can be ruled out as follows. In the experiments shown in Fig. 2, approximately 20–25 μg of total cellular DNA was loaded in each well. Because all our transformants contain the central 2.3-kb *Eco*RI fragment bearing *tk* sequences, 20–25 pg, at the very least, of this fragment is expected per copy per haploid genome. Because in our hands the photographic intensities obtained are linear in the range 5–100 pg of the 2.3-kb fragment, analysis of the photographic intensity of this fragment in digests of cellular DNA compared to that from standards (see Fig. 2) strongly suggests that there is one copy or less of the 2.3-kb fragment per haploid genome. Our results are in agreement with those of Pellicer *et al.* (10).

Stability of the Transformed Phenotype. The three transformants were grown in HAT medium, transferred into HT medium for five generations (approximately 7 days), and then transferred again to 2× medium. The relative plating efficiencies in BrdUrd as a function of generations in nonselective medium (2× medium) are shown in Fig. 3. A similar study was performed on transformant 1-2-12 after 120 and 190 generations in HAT medium. The percentage rate of loss of the tk$^+$ phenotype per generation, x, was calculated from $(1-x)^n = fsn$ in which n is the number of generations in nonselective medium and fsn is the fraction of BrdUrd-sensitive cells at n. The value for x was 0.6%, 2%, and 3% for 1-2-9, 1-2-12, and

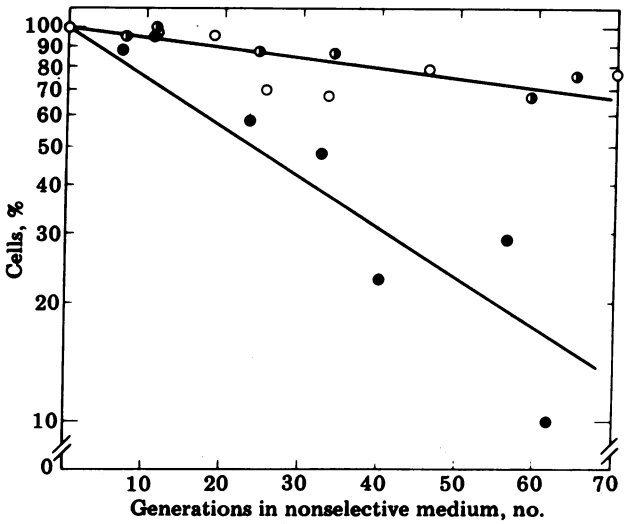

FIG. 3. Stability of the tk$^+$ phenotype. The percentage of cells leading to colony formation in 2× medium containing BrdUrd at 30 μg/ml divided by the percentage giving rise to colonies in 2× medium alone was subtracted from 100 and plotted against the number of generations in 2× medium (nonselective medium). Cells were plated in triplicate at the same time in both media. The absolute plating efficiencies were 50–80% for 1-2-9 and 1-2-15 and 20–35% for 1-2-12. ◑, Data for 1-2-9 after 100 generations in HAT medium; ○, for 1-2-9 after 170 generations in HAT medium; ●, for 1-12-15 after 90 generations in HAT medium.

1-2-15, respectively. This rate did not change appreciably for 1-2-9 and 1-2-12 in the two successive experiments.

The values obtained as shown in Fig. 3 were also checked by performing at least three determinations of plating efficiency in HAT medium and scoring for HAT resistance with LM cells as a control. For 1-2-9 and 1-2-12, the loss of HAT resistance paralleled the loss of BrdUrd sensitivity; this was not the case for 1-2-15. In LM cells, the tk^+ was not lost in 70 generations (i.e., $x < 0.01\%$). The values of x for the three transformants guarantee that, at any given time when the cells are harvested from HAT, at least 97% of them have a tk^+ phenotype.

The *tk* Sequences are Found in Nucleosomes. Nuclei from Ltk^-, 1-2-9, 1-2-12, and 1-2-15 cells were digested with staphylococcal nuclease and the purified DNA from the digests was run on agarose gels. The typical nucleosomal DNA ladder pattern obtained by staining the agarose gel with ethidium bromide is shown in Fig. 4*A*. When this DNA was transferred to a nitrocellulose filter and hybridized against the 3.5-kb *tk* fragment, the autoradiographic pattern shown in Fig. 4*B* was obtained. DNA obtained from Ltk^- $aprt^-$ nuclei did not contain any viral *tk* sequences. For the three transformed cell lines, the viral *tk* sequences were protected from nuclease attack in the same manner as the bulk host DNA. The unit size for the DNA ladder of the bulk nucleosomal DNA and the probed sequence was about 185 base pairs. It is highly unlikely that the chromatin bearing the *tk* sequences was created artifactually during the preparation of the nucleosomal DNA. In the low-ionic strength conditions used, neither nucleosomal dissociation and exchange (28) nor nucleosomal sliding (29) occurs.

DISCUSSION

The DNA of the HSV-1 *tk* gene in the nuclei of transformed cells is protected from staphylococcal nuclease in the same manner as bulk host cellular DNA, and we therefore infer that they are packaged in a similar fashion. Although there might turn out to be a family of structures that lead to the characteristic discrete pattern of protection from staphylococcal nuclease digestion, at present most of the experimental data point to a well-defined and relatively invariant structure, the nucleosome (30).

That the *tk* gene is packaged in nucleosomes is important for at least two reasons: (*i*) the *tk* gene is a foreign gene introduced into these cells, and (*ii*) this gene is being transcribed.

Investigations of the packaging of foreign genes introduced into the cells of higher eukaryotes have so far been limited to whole viral genomes found in the cell nucleus. The genomes that have been examined have been those of simian virus 40 (31), adenovirus (32, 33), HSV (34) and Epstein–Barr virus (35). Most, but not all, of these DNAs are covered by nucleosomes, and what determines whether or not these genomes are packaged into nucleosomes is still not known. Two facts, important to our discussion, are clear from these studies. (*i*) *A priori*, it is not possible to predict the structure of the nucleoprotein complex enveloping a foreign DNA introduced into an animal cell. (*ii*) Nucleosomal packaging *per se* is unrelated to the integration of the new sequences into the host genome (in all the cases studied the viral genome was in an unintegrated form).

Because the transformed (tk^+) phenotype is quite stable in HAT medium, it is almost a certainty that, as the cells are propagated, the HSV *tk* sequences are being actively transcribed.

Thus, for a gene coding for a product not restricted to terminally differentiated cells and which is therefore expressed, on the average, over several cell generations, transcriptional activity is not determined by the loss of nucleosomes. Our results support earlier work on hemoglobin and ovalbumin genes carried out with less precise and direct methods (36–38) but are different from the results obtained in a recent study that utilized the blotting technique to study the packaging of the heat-shock sequences in *Drosophila melanogaster* (39). Using this induc-

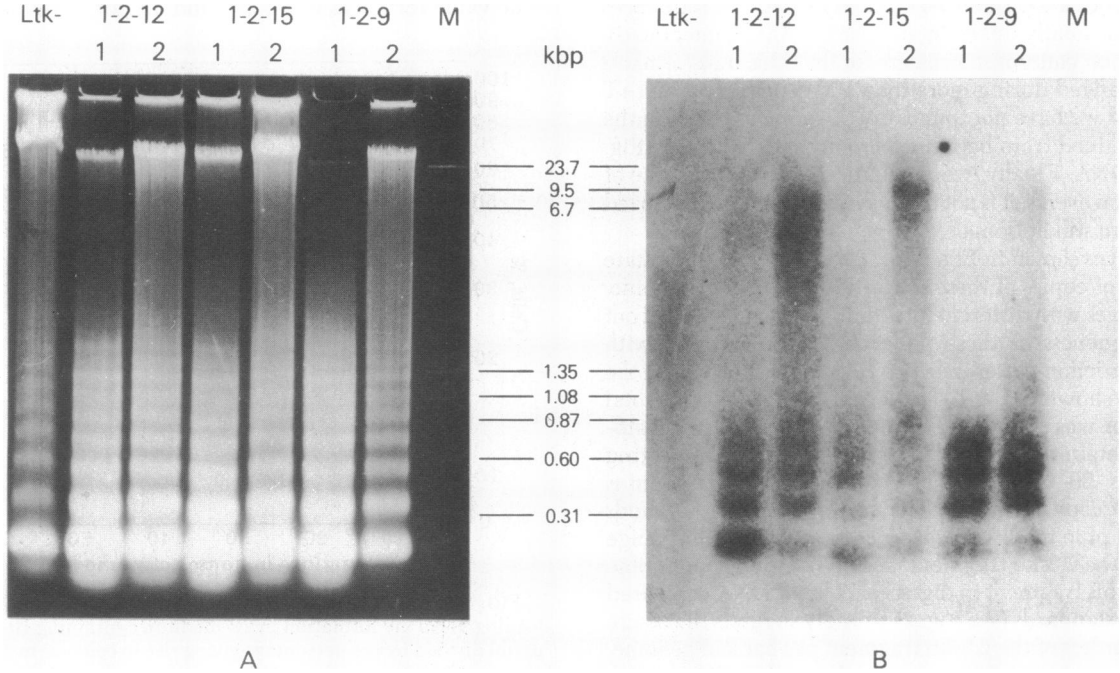

FIG. 4. Electrophoretic analysis of staphylococcal nuclease digests of nuclei from transformed and recipient cells, Ltk^- $aprt^-$ (designated at Ltk^- here). (*A*) DNA from staphylococcal digests of nuclei was isolated, electrophoresed, and stained with ethidium bromine (1 μg/ml in water). The extent of digestion for each sample (as percentage acid-soluble material) was as follows: Ltk^-, 3%; 1-2-12, 11.6% and 6.5% for lanes 1 and 2, respectively; 1-2-15, 12.7% and 5.5% for lanes 1 and 2; and 1-2-9, 10.2% and 4.5% for lanes 1 and 2. Lane M contained a mixture of a *Hin*dIII digest of bacteriophage λ DNA and an *Hae* III digest of bacteriophage φX174 DNA. (*B*) DNA from the gel was transferred to a nitrocellulose sheet and hybridized with a ^{32}P-labeled 3.5-kb *tk* probe. The exposure was for 10 days.

ible system, Wu *et al.* (39) showed that these genes are in nucleosomes when they are not expressed but not when they are actively transcribed. What accounts for the difference between our results and theirs is not clear.

The patterns in Fig. 4 also show that the repeat length of bulk cellular nucleosomes is indistinguishable from the repeat length for this actively transcribed sequence. Although this might not be true for certain multiple-copy genes, such as the 5S gene (40), our results do not support earlier suggestions that the repeat length might be related to the transcriptional level (41, 42). What makes such nucleosomes on active genes different from the rest, as shown by their preferential susceptibility to nucleolytic digestion by pancreatic DNase [DNase I (43)] and preferential excision by micrococcal nuclease (44), remains to be elucidated.

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