

# Rapid detection of ultraviolet-induced reversion of an amber mutation in mouse L cells

(mutagenicity testing/mammalian cells/transfection/mRNA stability)

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**ABSTRACT** An amber codon (TAG) was introduced into the N-terminal coding region of the murine *H-2K<sup>b</sup>* gene. The mutant gene was transfected into mouse L cells, and a clone containing a single unrearranged chromosomally integrated copy of the mutant gene was mutagenized with 254-nm UV radiation. Surviving cells were scored for surface expression of *H-2K<sup>b</sup>* protein with *in situ* immunoperoxidase staining. Revertants were detected at a frequency of  $3 \times 10^{-6}$  at a dose of 40 J/m<sup>2</sup> (3-5% survival). Revertant genes, cloned by plasmid rescue, contained the expected thymine-to-cytosine transitions at the amber codon. These data show that revertants can be rapidly detected in mammalian cells without selection and provide a basis for the development of mammalian cell lines that could be used to study mutational phenomena. During this study the steady-state level of mRNA was reduced in L cells carrying the amber mutant *H-2K<sup>b</sup>* gene compared with L cells containing a wild-type or revertant *H-2K<sup>b</sup>* gene. This reduction was shown not to be due to transcriptional differences, suggesting that the amber mutation decreases stability of the *H-2K<sup>b</sup>* mRNA.

Mutation and DNA repair have been the subject of intensive study over many years because of the fundamental role they play in the process of evolution and the development of genetic disease and cancer. A large amount of data pertain to the molecular basis of mutation in prokaryotic cells, but the greater complexity of the mammalian genome has limited a similarly detailed analysis of mutation in mammalian cells. The study of mutation in mammalian cells and mammalian cell mutation tests currently used to detect environmental mutagens all depend on the generation of forward mutations and selection of mutants by their resistance to drugs. Forward mutation tests are lengthy, taking 2-3 weeks, and although they correlate fairly well with *in vivo* carcinogenicity data (1), these tests are uninformative about the molecular nature of induced mutations. Recently, shuttle vectors have been used to rescue mutated genes from mammalian cells so that the genes can be analyzed genetically in bacteria (2-6). By DNA sequencing of bacterial *lacI* genes rescued in this manner, Calos and coworkers (2, 3) have shown that ethyl methanesulfonate and UV light each exhibit a mutational specificity that is similar in human and bacterial cells. For screening mutagens, however, this method has not yet proved practical, as it is time consuming and laborious.

In this paper we illustrate an alternative approach by the construction of a mammalian cell line that can be used to measure reversion of an amber mutation. This report represents a demonstration of unselected reverse mutation in mammalian cells, and it provides a basis for the development

of rapid mammalian reverse mutation systems that can be used in a manner analogous to the Ames bacterial reverse mutation test (7).

In principle, this mammalian system consists of a gene into which specific mutations are introduced such that the mutant gene will be transcribed but not correctly translated in mammalian cells. Mutant genes are introduced into a mammalian cell line by transfection, and stable clones expressing the mutant genes are then isolated; these stable clones are next treated with mutagen, and surviving cell populations are scored for cells expressing the protein product of the revertant gene. The number of revertants detected measures the mutagenicity of a given compound. By assaying directly for the revertant gene product, the requirement for a prolonged growth step is eliminated.

The gene chosen for this study was the mouse *H-2K<sup>b</sup>* gene, because it encodes a cell surface protein that is readily detectable by monoclonal antibodies (8). Revertant cells were detected using an *in situ* immunoenzymatic assay, and because this is most conveniently performed on cells that grow as a monolayer attached to the substratum, mouse L cells were chosen for this study. These cells have the added advantages of being readily transfectable and having a relatively rapid growth rate but, in principle, any adherent cell line could be used.

## MATERIALS AND METHODS

**Cell Culture and Transfection.** Mouse Ltk<sup>-</sup> cells were maintained in minimal Eagle's medium containing 10% newborn calf serum. Cells were transfected using calcium phosphate (9). Cotransfections were carried out using a 10-fold excess of nonselected DNA with respect to the plasmid pRT which carries the herpes simplex virus *tk* gene (10). Tk<sup>+</sup> colonies were selected and maintained in hypoxanthine/aminopterin/thymidine (HAT) medium. For analysis of transient *H-2K<sup>b</sup>* gene expression, cells were fixed with glutaraldehyde 48 hr after transfection and stained using an immunoenzymatic assay.

**Immunoenzymatic Staining.** Glutaraldehyde-fixed cells were incubated with the monoclonal antibody MV3 (anti-*H-2K<sup>b</sup>*) followed by a horseradish peroxidase-coupled goat anti-mouse IgG (Sigma). Color was developed using aminoethylcarbazole as a substrate.

**In Vitro Mutagenesis.** A 670-base pair (bp) *HindII/SmaI* fragment from the 5' end of the *H-2K<sup>b</sup>* gene (11) was subcloned into M13mp9. Oligonucleotide-directed site-specific mutagenesis was carried out as described by Zoller and Smith (12). After isolation of the amber mutant a 0.5-

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Abbreviations: RSV, Rous sarcoma virus; LTR, long terminal repeat.

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kilobase (kb) *Nde*I–*Hind*III fragment from pRSVcat (13) was cloned into the *Nru*I site upstream of the *H-2K<sup>b</sup>* gene. This fragment was then ligated to the remainder of the *H-2K<sup>b</sup>* gene to make a complete amber *H-2K<sup>b</sup>* gene.

**Hybridization Experiments.** Southern blots and S1 nuclease analysis of mRNA were carried out as described (14, 15). <sup>32</sup>P-labeled probes for Southern blotting were prepared by oligo-labeling (16).

**UV Mutagenesis.** Lam13 cells ( $5 \times 10^7$ ), growing semi-confluently in 15-cm dishes containing HAT medium, were exposed to 254-nm UV light at a dose of 40 J/m<sup>2</sup> after aspiration of the medium. [The UV dose was estimated by placing the UVX-25 sensor of a UVX radiometer (Ultraviolet Products, San Gabriel, CA) in the center of a 15-cm dish positioned 10 cm below a CAMAG 29200 Universal UV source.] The medium was replaced, and the cells were incubated overnight. The next day the cells were trypsinized and replated in 10-cm dishes. At this time samples were taken from control (untreated) and UV-irradiated cultures for estimation of cell survival by colony formation. Cells were allowed to recover for 2 days, then trypsinized, and replated at  $2 \times 10^6$  cells per 10-cm dish. Untreated Lam13 cells were plated at a similar density at the same time as control. Cells were then grown for 3 more days before fixing with glutaraldehyde and staining with the *in situ* immunoenzymatic assay.

**Transcriptional Run-Off Assays.** These were done as described by Linial *et al.* (17).

## RESULTS

**Construction of an L-Cell Line Containing an Amber Mutant *H-2K<sup>b</sup>* Gene.** An amber mutation was introduced into the 5' end of the *H-2K<sup>b</sup>* gene using oligonucleotide-directed site-specific mutagenesis. The mutation was introduced at residue 18 of the signal peptide by changing the glutamine codon, CAG, to the amber stop codon, TAG (Fig. 1). An additional silent base change was made at the third position of residue 19 to link the creation of a *Bss*HII site to the introduction of the amber mutation. The endogenous promoter of the *H-2K<sup>b</sup>* gene was replaced with a sequence from the Rous sarcoma virus (RSV) long terminal repeat (LTR), known to act as a strong promoter in a variety of mammalian cells (13), thus allowing efficient expression of the introduced mutant genes, as well as enabling them to be distinguished readily from endogenous *H-2K<sup>b</sup>* and other *H-2* sequences in hybridization experiments. This construct, henceforth referred to as the *H-2K<sup>b</sup>* amber gene, was cloned into the *Hind*II site of pUC19 and introduced into mouse L cells by cotransfection with the herpes simplex virus *tk* gene in the plasmid pRT (10). *Tk*<sup>+</sup> clones were selected in HAT medium and screened for expression of the *H-2K<sup>b</sup>* amber gene by S1 nuclease protection analysis of mRNA, with use of a probe specific for the 5' end of the *H-2K<sup>b</sup>* gene (Fig. 2 *Right*). Similar transfections were done with constructs containing the wild-type *H-2K<sup>b</sup>* gene under the control of the RSV promoter.

*Ltk*<sup>+</sup> clones expressing *H-2K<sup>b</sup>*-specific message were isolated from the *H-2K<sup>b</sup>* amber gene transfection (Fig. 2 *Left*). To determine whether the message produced by these cells was potentially capable of producing *H-2K<sup>b</sup>* protein, a genetic suppression test was carried out. Genetic suppression of amber mutations has been demonstrated using an *in vitro* mutagenized *Xenopus laevis* tyrosine tRNA gene in simian virus 40 vector (19, 20).

*Ltk*<sup>+</sup>/*H-2K<sup>b</sup>* amber clones were transfected with this plasmid, and cells were assayed for transient expression of *H-2K<sup>b</sup>* 48 hr after transfection using the *in situ* immunoenzymatic staining assay. All cell lines tested that produced *H-2K<sup>b</sup>*-specific message also yielded cells expressing *H-2K<sup>b</sup>* protein. In this assay the cells appeared to stain with a similar

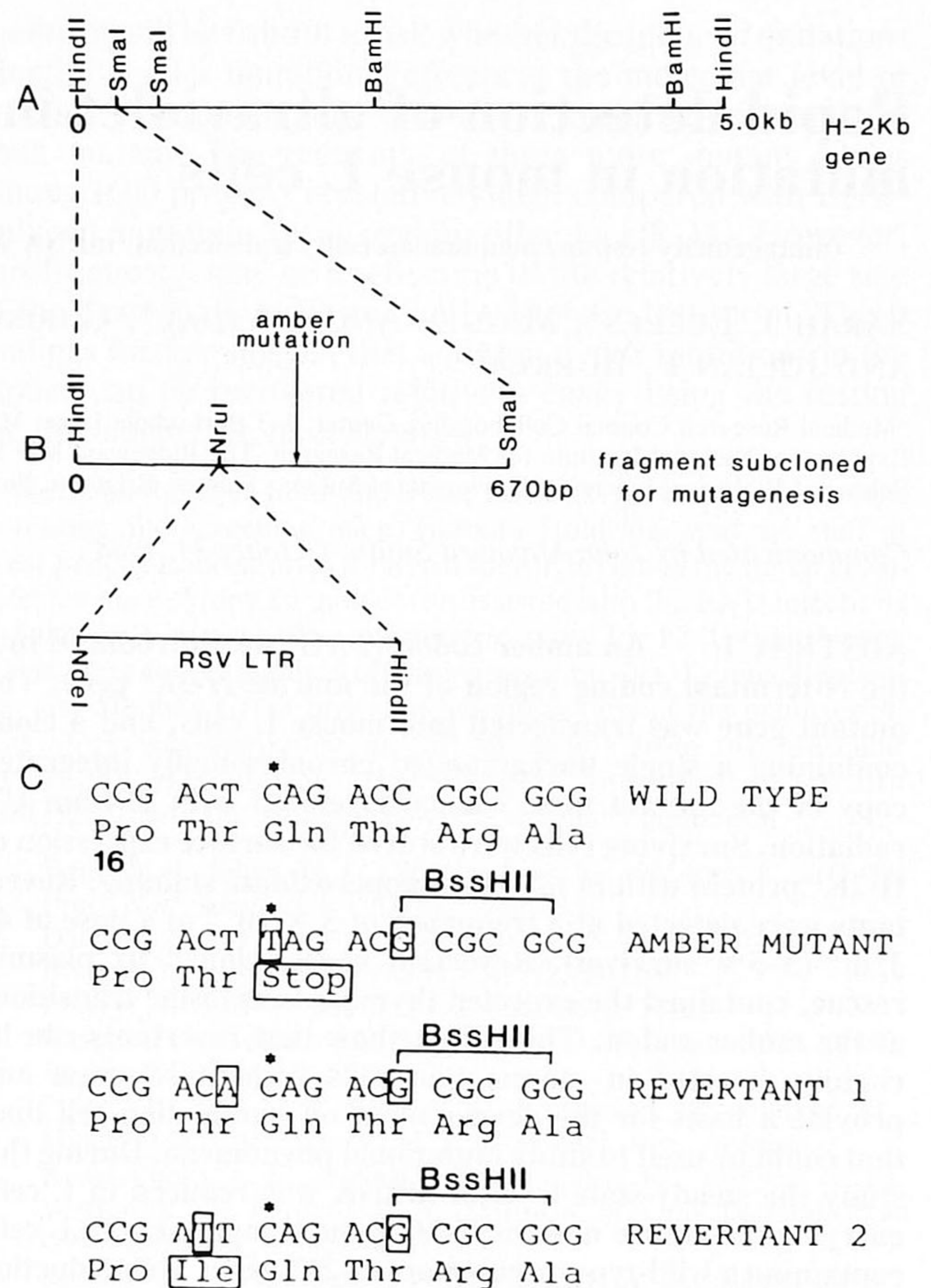


FIG. 1. Construction and sequence of mutant and revertant genes. (A) Restriction map of a 5-kb *Hind*II fragment carrying the *H-2K<sup>b</sup>* gene (11). (B) A 5' fragment of *H-2K<sup>b</sup>* gene subcloned in M13mp9 for oligonucleotide-directed site-specific mutagenesis. The position of the amber mutation and the site of insertion of the RSV LTR sequences are indicated. (C) Nucleotide sequences of the wild-type, amber, and revertant genes. Nucleotides and amino acids that differ from the wild-type sequence are boxed. \*, Position of the nucleotide that was altered to produce the amber mutation and that has reverted in both revertants.

frequency and intensity to that seen when L cells are transfected with the wild-type *H-2K<sup>b</sup>* gene. Stability of the L-cell clones was assessed by repeated isolation of RNA followed by S1 nuclease protection analysis; clones containing the wild-type gene were stained periodically using the immunoenzymatic assay. Under these criteria all clones used in this study were stable for at least 4 months when grown in HAT medium.

Southern blots were done to determine the copy number of the integrated *H-2K<sup>b</sup>* amber genes using a 500-bp *Nde*I–*Hind*III fragment from pRSVcat, containing RSV LTR sequences, as a probe (13). Copy number was estimated by comparison with known amounts of the input (pUC19 *H-2K<sup>b</sup>* amber) plasmid DNA (Fig. 3). The copy number varied between 1 and  $\approx 10$  copies per genome. Although it contained several rearranged copies of plasmid sequences, clone Lam13 was selected for mutagenesis because it appears to contain a single unrearranged copy of the *H-2K<sup>b</sup>* amber gene (Fig. 3). The unrearranged copy is probably flanked by plasmid sequences that should allow the intact input plasmid to be rescued from Lam13 after mutagenesis.

**UV Mutagenesis and Identification of Revertants.** Lam13 cells were mutagenized by treatment with 254-nm UV light. After a 2-day recovery period the surviving cells were trypsinized and replated at  $2 \times 10^6$  cells per 10-cm dish. Untreated Lam13 cells were plated at a similar density at the

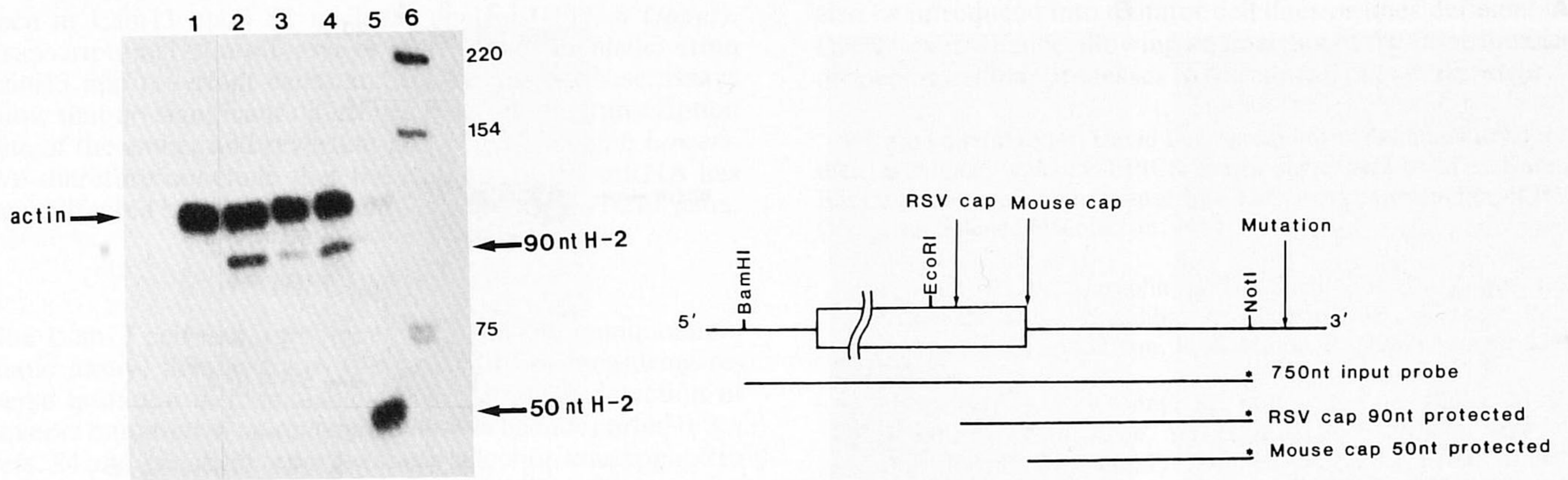


FIG. 2. S1 protection analysis of mRNA from L-cell clones containing the *H-2K<sup>b</sup>* amber gene. (Left) Ten micrograms of total cytoplasmic RNA was hybridized to a <sup>32</sup>P 5'-end-labeled 750-bp *Bam*HI–*Not* I fragment from the 5' end of the *H-2K<sup>b</sup>* gene together with a <sup>32</sup>P 5'-end-labeled 300-bp *Bam*HI–*Ava* I fragment including the 5' end of a human actin cDNA clone (18). S1 nuclease analysis was carried out as described (15). Arrows indicate the position of *H-2K<sup>b</sup>* and actin-protected fragments. Lanes: 1, L cell RNA; 2–4, RNA from L-cell clones containing *H-2K<sup>b</sup>* amber genes (lane 3 represents clone Lam13); 5, B6 mouse liver RNA; 6, pBR322 *Hin*fl size markers. (Right) The 5' end of the *H-2K<sup>b</sup>* gene, indicating the probe and protected fragments originating from the RSV and *H-2K<sup>b</sup>* cap sites, respectively. \*, Position of the <sup>32</sup>P-labeled end.

same time as a control. Cells were allowed to grow for 3 more days before fixing with glutaraldehyde and analysis with the immunoenzymatic staining assay. The results of two such experiments are shown in Fig. 4. Revertants were scored using an inverted microscope. Because DNA replication is required for fixation of mutations, cells were scored as revertants only where more than one adjacent cell was stained; this eliminates the possibility of including artifacts arising from nonspecific staining of individual dead cells, and for this reason the 3-day growth step was included in the protocol. The frequency of revertants induced by mutation

with UV light in the surviving cell population was  $3 \times 10^{-6}$  at a total dose of 40 J/m<sup>2</sup>. No spontaneous revertants were seen in the untreated cells (Fig. 4).

**Isolation of Revertant Genes.** To confirm that cells stained in the *in situ* assay did, indeed, represent revertants, the revertant population was purified and *H-2K<sup>b</sup>* genes were isolated so that the nucleotide sequence of the region of interest could be determined. A population of Lam13 cells was treated with UV as described, and the surviving cells were grown for 5 days and then labeled with the monoclonal antibody MV3 (anti-*H-2K<sup>b</sup>*) followed by a fluorescein isothiocyanate-conjugated goat anti-mouse second antibody and analyzed using an EPICS V fluorescence-activated cell sorter. Propidium iodide (10 μg/ml, final concentration) was added to the cells before analysis, so that dead cells that exhibit red fluorescence could be gated out. The EPICS sorter was set to collect the top 3% of cells exhibiting green fluorescence, and the resulting cells were recultured; this procedure was repeated twice. Samples of the population were subjected to *in situ* analysis after each sort, and after three sorts ≈70% of the population consisted of *H-2K<sup>b</sup>*-expressing cells (Fig. 5B).

DNA was prepared from the enriched population and from untreated Lam13 cells, and the *H-2K<sup>b</sup>* genes were cloned by

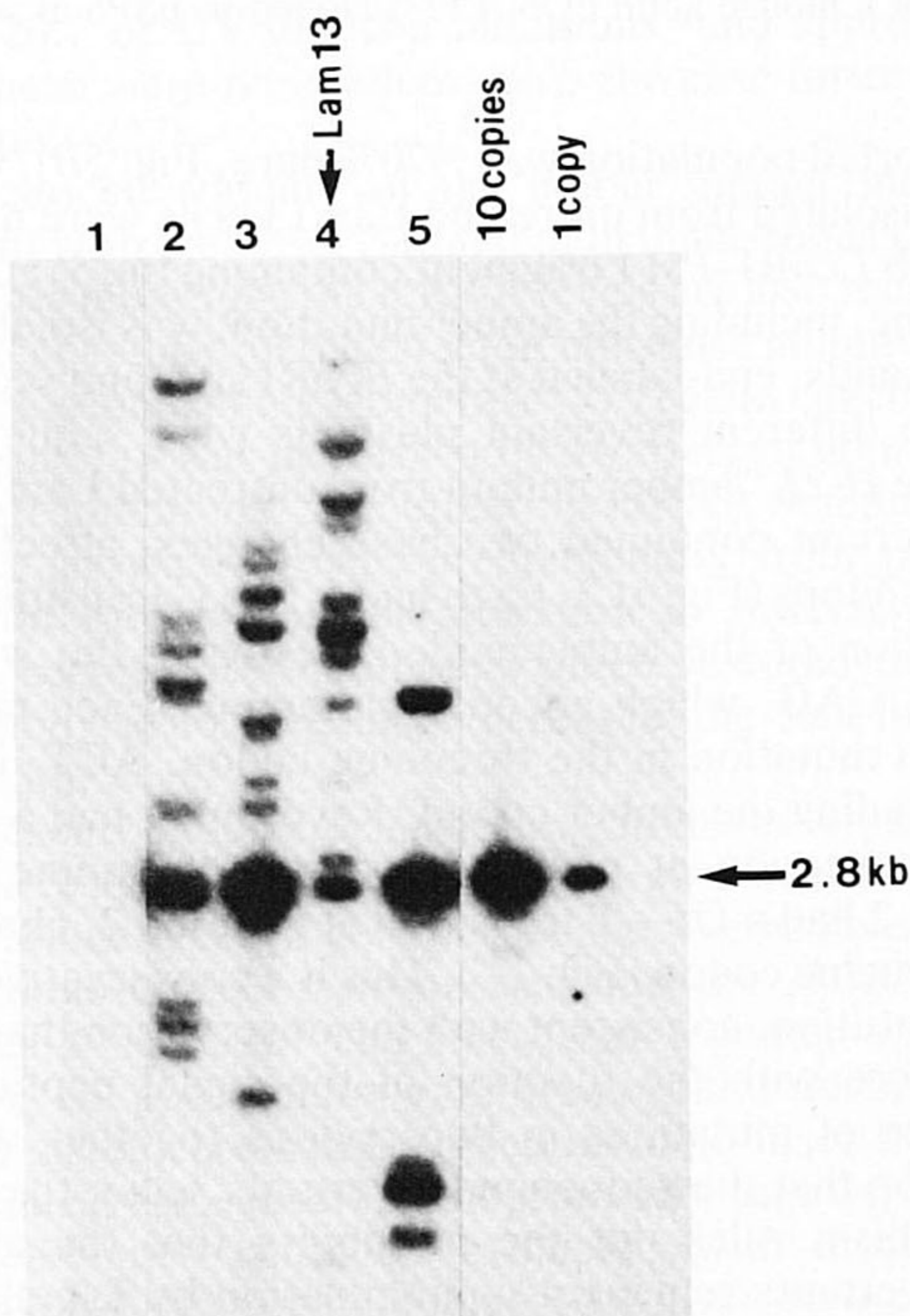


FIG. 3. Southern hybridization analysis of L-cell clones containing *H-2K<sup>b</sup>* amber genes. Five micrograms of *Bam*HI-restricted DNA from L cells (lane 1) or from L-cell clones expressing *H-2K<sup>b</sup>* amber mRNA (lanes 2–5) was loaded per lane. A <sup>32</sup>P-oligo-labeled 500-bp *Nde* I–*Hind*III fragment from pRSVcat (13) was used as a probe. This probe hybridizes to a 2.8-kb *Bam*HI fragment extending from the *Bam*HI site in the pUC19 polylinker through the RSV sequences to the *Bam*HI site in the middle of the *H-2K<sup>b</sup>* gene (Fig. 1). Either 100 or 10 pg of *Bam*HI-digested pUC19 *H-2K<sup>b</sup>* amber plasmid DNA was mixed with 5 μg of *Bam*HI-digested L-cell DNA and loaded as standards, being the equivalent of 10 and 1 gene copies, respectively.

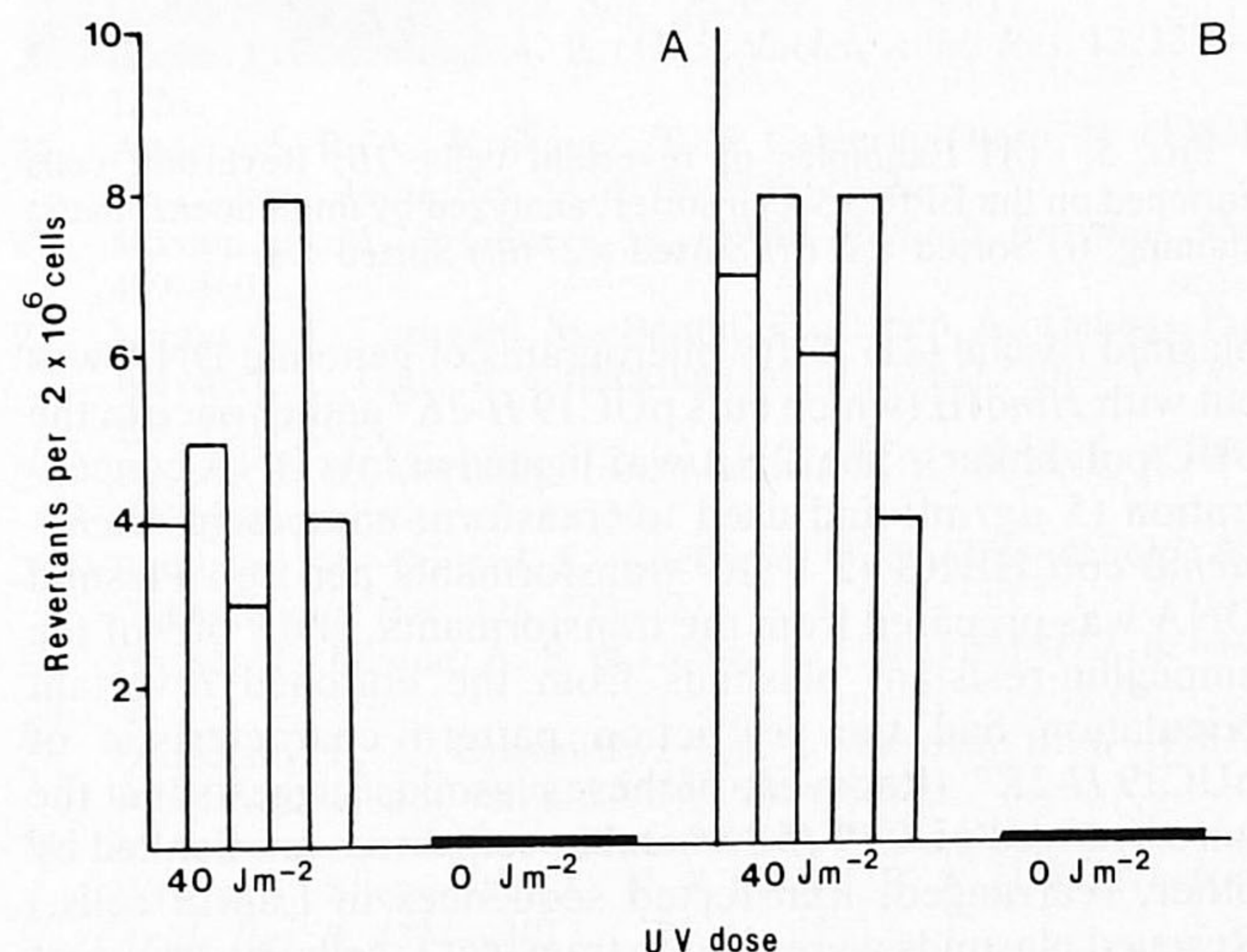


FIG. 4. UV mutagenesis of Lam13 cells. (A and B) Results of two independent mutagenesis experiments. Revertant cells were scored as described in the text. Each bar represents the number of revertants scored per  $2 \times 10^6$  surviving cells.

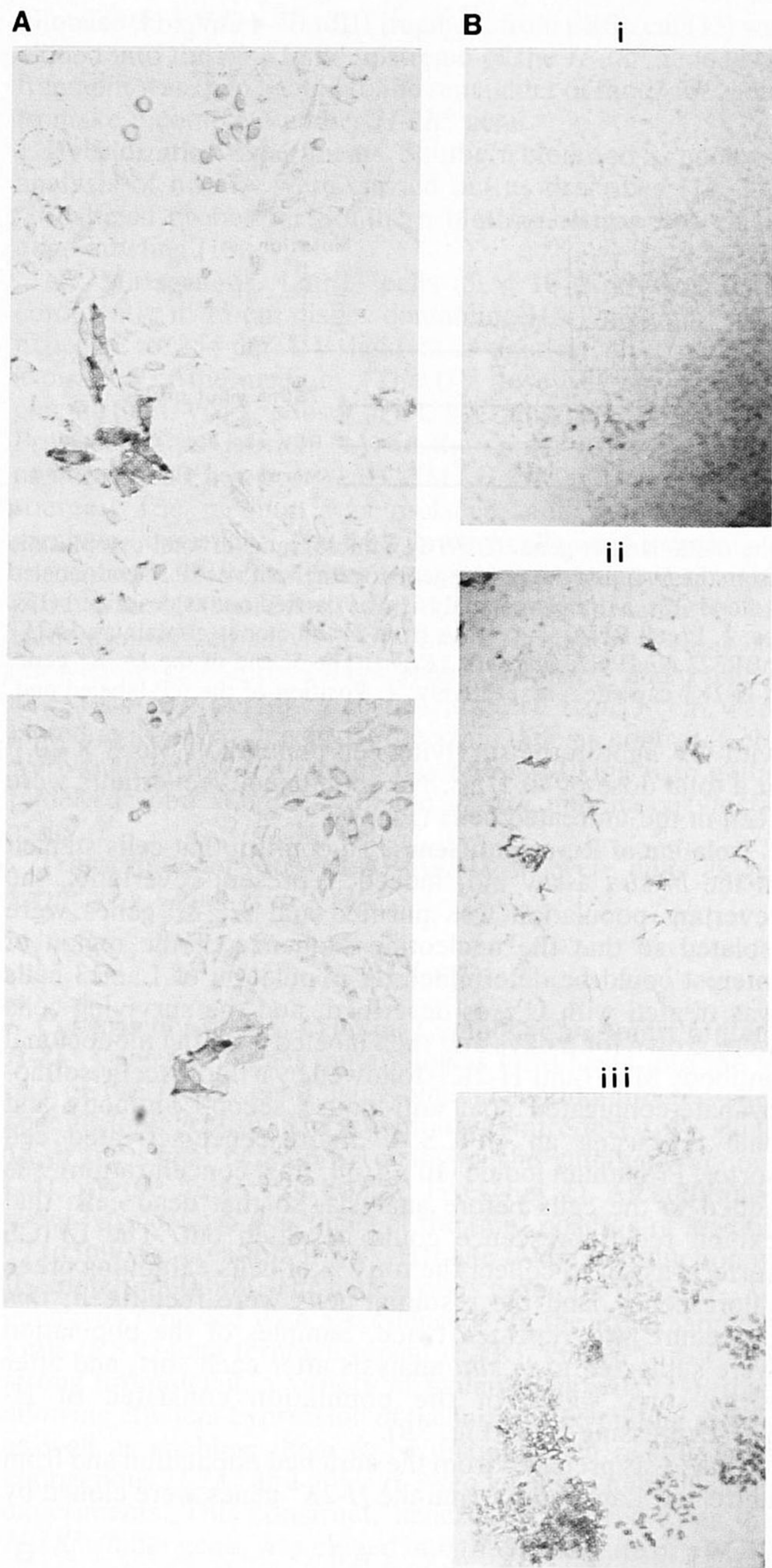


FIG. 5. (A) Examples of revertant cells. (B) Revertant cells enriched on the EPICS V cell sorter, analyzed by immunoenzymatic staining: (i) Sorted  $\times 1$ . (ii) Sorted  $\times 2$ . (iii) Sorted  $\times 3$ .

plasmid rescue (21). Fifty micrograms of genomic DNA was cut with *Hind*III (which cuts pUC19 *H-2K<sup>b</sup>* amber once in the pUC polylinker). The DNA was ligated at low DNA concentration (5  $\mu\text{g}/\text{ml}$ ) and used to transform competent *Escherichia coli* HB101 ( $2 \times 10^7$  transformants per  $\mu\text{g}$ ). Plasmid DNA was prepared from the transformants, and 7 of 9 of the ampicillin-resistant plasmids from the enriched revertant population had the restriction pattern characteristic of pUC19 *H-2K<sup>b</sup>*. (Recovery of these plasmids suggests that the unrearranged pUC19 *H-2K<sup>b</sup>* amber sequences are flanked by other, rearranged, transfected sequences in Lam13 cells.) Rescued plasmids were used to transfect L cells in a transient assay. Forty-eight hours after transfection the cells were stained for expression of *H-2K<sup>b</sup>*, and 4 of 7 plasmids that had the pUC19 *H-2K<sup>b</sup>* restriction pattern yielded transient transfectants expressing *H-2K<sup>b</sup>* (consistent with the observation

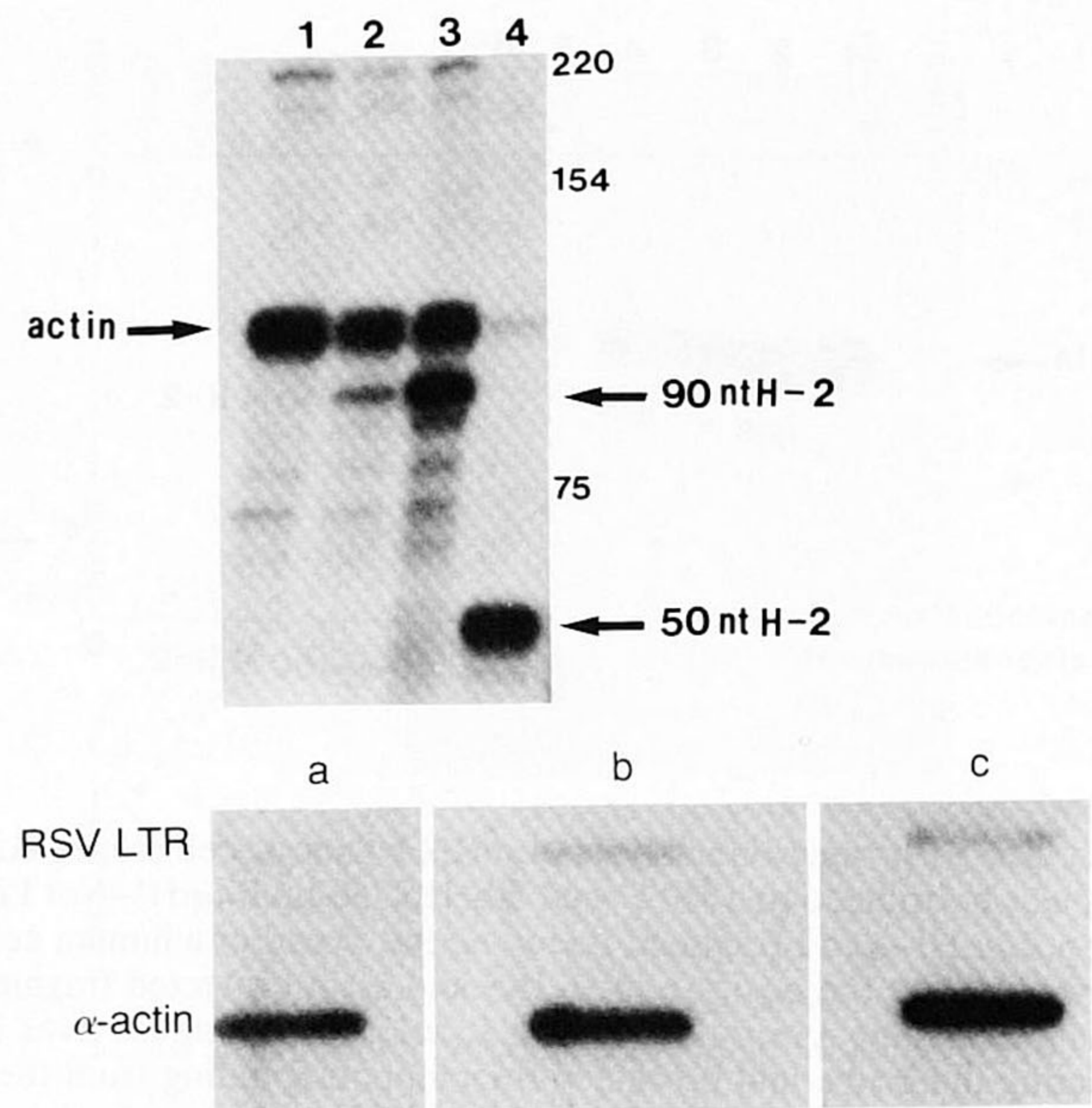


FIG. 6. (Upper) S1 nuclease analysis of mRNA from mutant and revertant cells. S1 nuclease analysis was done as described for Fig. 2. Lanes: 1, L-cell RNA; 2, Lam13 RNA; 3, RNA from the revertant population; 4, B6 mouse liver RNA. The position of migration of the protected *H-2K<sup>b</sup>* and actin fragments is indicated. (Lower) Transcriptional run-off analysis of mRNA from mutant and revertant cells. Nuclei isolated from L cells (a), Lam13 cells (b), and the enriched revertant population (c) were incubated with [ $^{32}\text{P}$ ]UTP for 30 min at 37°C. RNA extracted from these nuclei was hybridized to nitrocellulose filters on which the following DNAs had been immobilized: 1  $\mu\text{g}$  of a 500-bp *Nde*I-*Hind*III fragment carrying the RSV LTR, to detect *H-2K<sup>b</sup>* transcripts arising from the transfected gene and 2  $\mu\text{g}$  of a mouse actin cDNA (23) cloned in pSP6 as a positive control.

that the sorted population was  $\approx 70\%$  pure, Fig. 5B), whereas plasmids isolated from untreated Lam13 cells were negative.

A 1.4-kb *Eco*RI-*Pst*I fragment containing the 5' end of the *H-2K<sup>b</sup>* gene, including the amber mutation, was isolated from these plasmids, end-labeled at the *Eco*RI site, and sequenced (22). Two different revertant plasmids were sequenced as well as the *H-2K<sup>b</sup>* amber mutant from untreated Lam13 cells. Each revertant contained two base changes, affecting two different codons (Fig. 1C). Each had a T  $\rightarrow$  C transition in the first position of the amber codon, restoring the wild-type sequence, CAG, which encodes glutamine. Each revertant also had a mutation in the threonine codon, ACT, immediately preceding the amber codon. Revertant 1 had a silent T  $\rightarrow$  A transversion at position 3 of the threonine codon. Revertant 2 had a C  $\rightarrow$  T transition at position 2, changing it to an isoleucine codon (Fig. 1C). This is a conservative amino acid substitution, consistent with the observation that it does not interfere with the function of the signal peptide. The occurrence of mutations in two codons together with the observation that the sequenced revertants retain the *Bss*HII polymorphism rules out the possibility that the apparent amber revertants represent contamination by L cells transfected with the wild-type gene.

**Effect of the Amber Mutation on mRNA Stability.** During development of the Lam13 cell line we noted that L-cell clones containing the *H-2K<sup>b</sup>* amber gene produced  $\approx 10$ -fold less *H-2K<sup>b</sup>*-specific mRNA than similar clones transfected with the wild-type gene. This result suggested that lower levels of mRNA might result from a shorter mRNA half-life due to the introduction of the stop codon shortly after the start of translation. If so, the mRNA levels in the revertants should have increased. Indeed, mRNA from the revertant population showed a 10- to 20-fold increase over the level

seen in Lam13 upon S1 nuclease analysis (Fig. 6 Upper). Transcriptional run-off assays were done on nuclei from Lam13 and revertant cells, and the results of these assays show that no significant difference exists in the transcription rate of the amber and revertant *H-2K<sup>b</sup>* genes (Fig. 6 Lower). We therefore conclude that the stability of the mRNA has been affected by the amber codon in the mutant *H-2K<sup>b</sup>* gene.

## DISCUSSION

The Lam13 cell line, in conjunction with the immunoenzymatic assay, demonstrates the feasibility of measuring reverse mutation in mammalian cells. Although detection of reverse mutation in mammalian cells has been reported (e.g., refs. 24 and 25), in all reported cases selection was applied to the cells—thus introducing the possibility of selecting for mutations at other loci. Indeed, the spontaneous and induced frequencies of reversion reported in these studies suggests that not all measured events represented true reversions. The actual spontaneous reversion frequency of the *H-2K<sup>b</sup>* amber gene in Lam13 was not determined in this study, but it was  $<5 \times 10^{-8}$  (Fig. 4). UV-induced mutations were detected at a frequency of  $3 \times 10^{-6}$  with this cell line. The sensitivity of detection could be increased by using a cell line containing multiple copies of the mutant gene.

The revertants isolated in this study each contained the expected T → C transition at the amber codon as well as an additional mutation. All mutations occurred at the pyrimidine triplet sequence CTT, suggesting the probable involvement of either (6–4)pyrimidine/pyrimidone or cyclobutane dimers in the generation of these mutations (26, 27). Double mutations are frequently induced by UV; for example, a recent study of UV-induced mutation at the *APRT* locus in Chinese hamster ovary cells demonstrated that double mutants constituted 20% of UV-induced mutations, and approximately half of these were nontandem, as is the case for revertant 2 in this study (27).

The reduced stability of the amber mutant message is consistent with the observation that in thalassemias resulting from amber mutations the mRNA levels are also reduced (28, 29). A recent study on the effect of nonsense mutations in the human triosephosphate isomerase (*TPI*) gene concluded that mRNA stability was decreased by a variety of nonsense and frameshift mutations. The effect on mRNA stability was in this case shown to result predominantly from premature translation rather than an alteration in mRNA structure because second-site mutations, which restored translation termination to normal, also increased the half-life of the mRNA (30).

At present, mutagenicity testing involves conducting a series of tests on different genes or chromosomes, which assess various types of genetic damage. We believe a variety of mutations could be introduced into the *H-2K<sup>b</sup>* gene to construct a series of cell lines that together will detect reversion of point mutations, frameshifts, recombination between sister chromatids, deletions, and insertions in the same gene. By use of the rapid procedure that we described here these lines could be evaluated with a number of mutagens and the results compared with *in vivo* carcinogenicity data. We predict that tests based on this principle would complement the Ames test by detecting some mutagens that may not be detected in prokaryotes because of the fundamental differences in organization of DNA and in metabolism of bacterial and mammalian cells. Furthermore, this system could be used to study some basic aspects of cell biology. For instance, cell lines could be constructed containing mutant genes integrated at different sites in the genome to assess the influence of the site of integration on the frequency and spectrum of mutations. Mutant genes could

also be introduced into mutator cell lines or lines deficient in DNA repair—hence allowing an analysis of the contribution of specific cellular processes to the control of genetic fidelity.

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