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Differences between Rodent and Human Cell Lines in the Amount of Integrated DNA after Transfection

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The suitability of Chinese hamster and human cell lines for DNA-mediated gene transformation was investigated with respect to two parameters: the average quantity of and the integrity of integrated exogenous DNA fragments. No large differences were observed between most cell lines concerning the extent of fragmentation of the transferred DNA molecules. By contrast, the average number of sequences stably incorporated by the human cells (four lines tested) was 20- to 100-fold lower than the average amount inserted in the five Chinese hamster lines investigated. The very low uptake exhibited by the human cells, ranging from less than 100 up to 500 kb, renders these cells less suitable for transfection with genomic DNA to isolate specific genes.

A commonly used strategy for cloning specific genes involves DNA-mediated gene transfer using genomic DNA with or without added dominant marker genes (for reviews, see [1, 2]). Transformants that have incorporated the gene of interest can be isolated by specific selection systems [3–7]. In general, a second round of transfection is necessary to reduce the number of irrelevant co-integrated DNA sequences [5, 8]. Following cloning of DNA of such a transformant in *Escherichia coli,* the transferred gene can be identified and subsequently isolated in various ways ([5, 6, 9–11]). For most genes cloned using these procedures, rodent cells (Ltk$^-$, NIH3T3, CHO) have been employed as recipients for the transfection.

Following this strategy we have cloned a human gene designated *ERCC-1* that corrects the excision repair defect of an ultraviolet (UV) and mitomycin-C sensitive CHO mutant [11]. Extensive efforts to clone in the same way other human excision repair genes, complementing the excision repair defects of cells from xeroderma pigmentosum (XP) patients, have failed [11, 12]. This prompted us to compare CHO, XP and other cells with respect to the average quantity and integrity of exogenous DNA stably inserted in the genome. The findings in this study revealed general criteria that are important for determining whether specific cell lines are suitable for genomic DNA transfection.

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MATERIALS AND METHODS

Cell Lines

Nine cell lines were used as recipients for transfection. Five of these were of CHO origin: 43-3B and 27-1 are excision repair mutants derived from the CHO-9 subline [13], CHO 12RO is a UV-sensitive mutant from the CHO subline [14]. CHO and CHO-9 have been grown separately for many years. The four (human) cell lines tested were HeLa (epithelioid) and three SV40-transformed xeroderma pigmentosum (XP) fibroblasts: XP 12RO-SV [15], XP 2OS-SV [16] both belonging to XP complementation group A, and XP 2YO-SV (XP-F) [17]. The cells were grown in F10/DMEM (1 : 1) medium supplemented with 7% fetal bovine serum (FBS), and antibiotics.

Isolation Transfectants

About 5×10^5 CHO or 8×10^5 human cells were seeded in 100-mm Petri dishes one day before DNA transfection. DNA of cosmid 43-34 containing the two dominant marker genes xanthine–guanine phosphoribosyltransferase (*gpt*) and aminoglycoside 3′-phosphotransferase (*agpt*) and the human excision repair gene *ERCC-1* ([11], fig. 1) was digested with the restriction enzyme Cla I and mixed with genomic mouse DNA in a ratio of 1 : 3 on mass basis. The mouse DNA was digested with Taq I, which generates sticky ends that are complementary to those Cla I. DNA was offered to the cells in a calcium phosphate co-precipitate [18].

Two μg of linearized cosmid 43-34 and 8 μg of mouse DNA were added to each Petri dish. Following exposure of the DNA to the cells (overnight for CHO and 6 h for the human cells), cells were treated with dimethyl sulfoxide (DMSO) (10% for 30 min) and grown for 24 h in normal culture medium. Selection for *E. coli gpt* was in medium supplemented with aminopterin (0.2 μg/ml); thymidine (5 μg/ml); xanthine (10 μg/ml); hypoxanthine (15 μg/ml); mycophenolic acid (25 μg/ml) and deoxycytidine (2.3 μg/ml) [11, 19]. Selection for *agpt* was in medium containing the antibiotic G418 (HeLa cells in 500 μg/ml and XP cells in 300 μg/ml) [20, 21]. The selection media were replenished every 3–4 days. Distinct clones are usually visible 10–14 days after transfection. Southern blot analysis and in situ hybridization of transfected sequences in individual clones as well as mass populations have shown that the exogenous sequences in selected transformants become integrated in the genome of the host cell. We have failed to observe any significant loss of integrated sequences when selected transformants are cultured in the absence of selecting agents (unpublished observations). Nevertheless, cells were grown in selection medium up to the time of DNA isolation (usually 2 weeks after appearance of clones) except when stated otherwise.

DNA Procedures

The methods used for genomic DNA preparation, restriction enzyme digestion, agarose gel electrophoresis and Southern blot hybridization were performed essentially as described [11, 20, 22, 23].

RESULTS

To obtain information on the average amount of and integrity of exogenous DNA integrated into the genome of a large number of transformants, the following transfection experiment was performed. DNA of cosmid 43-34 containing two dominant marker genes and the human excision repair gene *ERCC-1* (fig. 1) was digested with the restriction enzyme Cla I, mixed with Taq I-digested mouse genomic DNA in a ratio of 1 : 3 on a mass basis and offered to the cells in a calcium phosphate co-precipitate. Five CHO and four human cell lines were used as recipients for transfection.

After transfection, the transformants were selected for the uptake of one of the the two dominant marker genes present on cosmid 43-34 (fig. 1). All Chinese

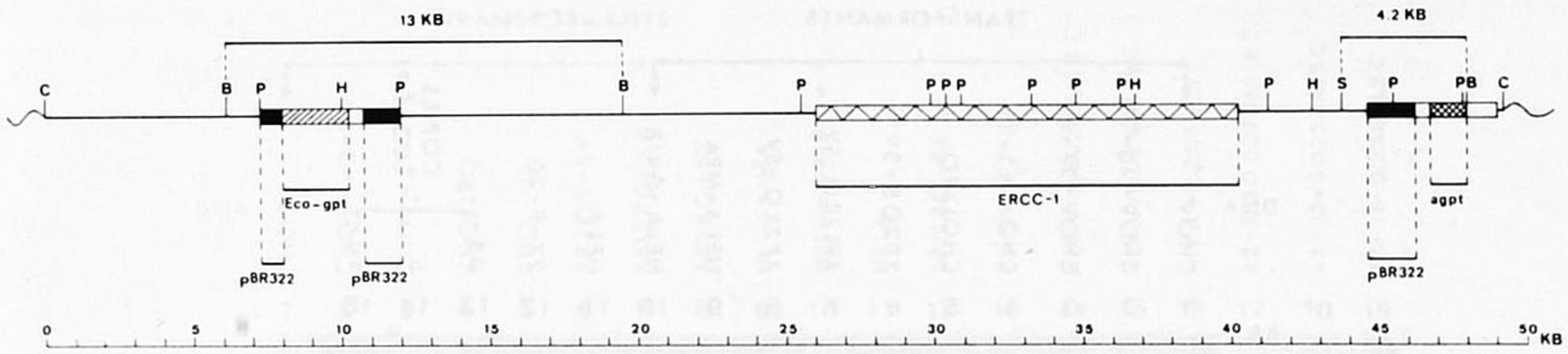

Fig. 1. Physical map of cosmid 43-34. Cosmid 43–34 contains two dominant marker genes: the *agpt* gene encoding G418 resistance [21] and the *E. coli gpt* gene which affords resistance against mycophenolic acid (MPA) [19]. In addition, it harbors the 15-kb human excision repair gene *ERCC-1* [11]. The position of the genes and pBR sequences is indicated. Symbol designation of the restriction endonuclease cleavage sites: *B*, Bgl II; *C*, Cla I; *H*, Hind III; *P*, Pst I; *S*, Sal I. For clarity, only relevant sites are given.

hamster cells were selected for the uptake of the *E. coli gpt* gene using mycophenolic acid as the selecting agent (transfection frequency 10^{-3}–5×10^{-3}).

The human cells were selected for G418 resistance (uptake of the *agpt* gene) (transfection frequency approx. 10^{-4}–10^{-5}). This transfection frequency is at most one-tenth of that found in the Chinese hamster cells. However, selection for MPA resistance of the human cells resulted in even lower numbers of transformants, presumably due to metabolic cooperation (data not shown). To compare both selection protocols, HeLa cells were selected separately on both agents. Transformants were obtained within 2–3 weeks after transfection. DNA was isolated from the pooled population consisting of 200–>3 000 independent transformants of each cell line and characterized by Southern blot analysis using *gpt*, *agpt* and mouse genomic DNA as probes.

The results of the *gpt* and *agpt* hybridizations are shown in fig. 2*A*, *B*. The constant band in each lane (4.4 kb in fig. 2*A* and 2.3 kb in fig. 2*B*) represents the Pst I fragment harboring the respective gene (see also fig. 1). The hybridization signal for different numbers of *gpt* copies per genome is titrated in fig. 2*A*, lanes *13–15*. Fig. 2 shows large differences in the mean number of copies of these genes integrated into the genome of the different cells. The CHO 9 group of cells (CHO-9, 43-3B and 27-1) exhibit the strongest hybridization intensity, followed by the other two related CHO lines (CHO, CHO 12RO).

The HeLa cells and, particularly, the SV40-transformed XP fibroblasts display much weaker signals. From the intensity of hybridization in the transformant 27-9-2d which contains one intact *gpt* copy (fig. 2*A*, *12*) and the titration for the *gpt* copy number per diploid genome in the fig. 2*A*, *13–15* we estimate that the XP transformants harbor on the average less than four copies of each dominant marker per cell. (The greater intensity of the 4.4 kb band seen in fig. 2*A* with XP2OS-SV (fig. 2*A*, *6*) and XP2YO-SV (fig. 2*A*, *8*) is at least in part due to the fact that the probe contains SV40 sequences which also hybridize to multiple SV40 copies in the genome of these SV40-transformed cells, causing extra bands (triangles) and an overall increase in background (see caption to fig. 2 for

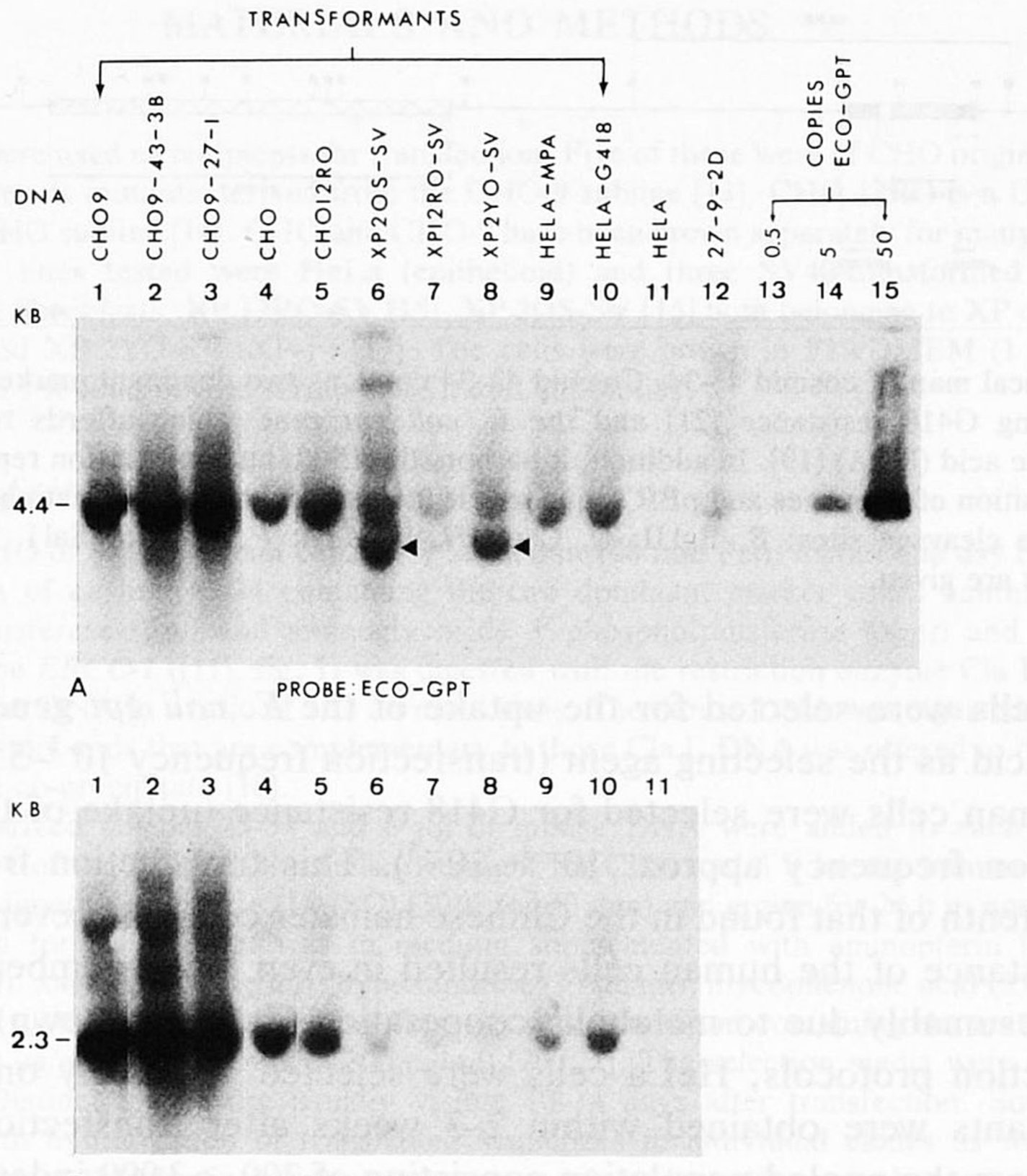

Fig. 2. Southern blot analysis of DNA of CHO and human transformed cell populations: determination of the average amount of integrated dominant marker gene copies. Stable transformants obtained after selection for the uptake of dominant marker genes were grown as mass population. DNA of the pooled transformants (derived from 200–>3 000 clones) of every cell line was extracted and digested with restriction endonuclease Pst I. Care was taken to layer in every lane the same amount of DNA (15 μg). After electrophoresis through a 0.6% agarose gel, the DNA was transferred to nitrocellulose and the filter hybridized with a ^{32}P-labelled *gpt* probe (*A*). Since this probe is an Sph I fragment isolated from the pSV3*gpt* vector [19] it also contains SV40 sequences [11]. The latter hybridize to the multiple copies of the endogenous virus genome in two of the three SV40-transformed XP fibroblasts yielding additional bands (e.g., fragments indicated by a triangle) and an overall increase of background. (The third line, XP12ROSV, contains only 1.5 copy of SV40 in the genome.) After removal of the bound radioactivity by alkali treatment the same filter was rehybridized with a ^{32}P-labelled *agpt* probe (*B*). The *agpt* was a 2 kb *EcoR* I fragment of plasmid pMCS [29] containing the *agpt* gene. The cell lines are as indicated. *HeLa-G418, HeLa-MPA:* HeLa transformants obtained after selection for G418 and MPA respectively, 27-9-2d. CHO 12RO, transformant containing one intact copy of the *Eco gpt* gene. Lanes *13–15:* HeLa DNA (1 μg) to which was added plasmid pSV3*gpt*H, linearized by Pst I in amounts equivalent to 0.5, 5 and 50 copies per diploid genome.

details).) The CHO-9-related cells have probably in the order of 100 copies per transformant. Furthermore, the HeLa cells selected for *gpt* uptake display for both dominant markers approximately the same hybridization level as for the HeLa transformants subjected to the G418 selection regime. This shows that the selection system does not significantly influence the number of dominant marker

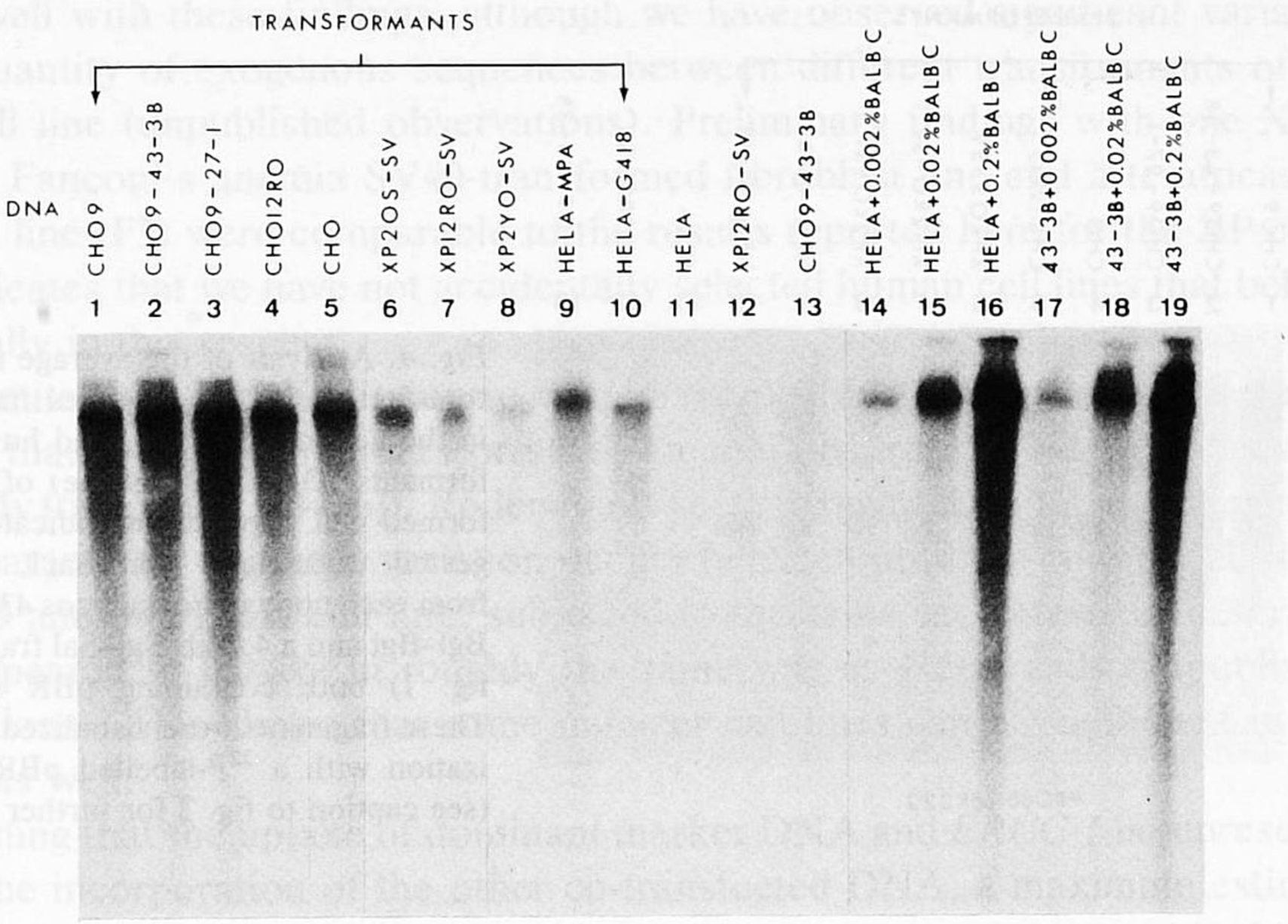

Fig. 3. Southern blot analysis of the average amount of mouse carrier DNA integrated in the genome of pooled transformants of CHO and human cell lines. DNA (10 μg/lane) of the pooled transformants of the cell lines indicated and of non-transformed control cells was digested with Hind III and hybridized with ^{32}P-labelled mouse genomic DNA. Lanes *14–16*, *17–19:* Titration of mouse DNA added to HeLa or 43-3B DNA (10 μg/lane) respectively. (See caption to fig. 2 for further details.)

genes inserted and that approximately equal numbers of *gpt* and *agpt* copies are integrated. This finding was confirmed by the fact that a large proportion (60%, up to more than 90%) of the HeLa as well as XP transfectants selected on G418 (or MPA) proved to be cross-resistant to the other selecting agent. Results similar to those for *gpt* and *agpt* uptake were obtained when the Southern blot analysis was carried out for the third gene present on cosmid 43-34, the human excision repair gene *ERCC-1* which is 15 kb (fig. 1, data not shown). Furthermore, analysis of individual CHO, HeLa and XP transformants yielded results consistent with the picture emerging from the analysis of the mass populations presented in fig. 2, although considerable differences were found between different transformants of the same cell line (unpublished observations).

Comparable relative hybridization intensities are also observed when the pooled transformants are analysed for the amount of mouse carrier DNA integrated using total mouse DNA as probe (fig. 3). From fig. 3, *14–19* in which increasing amounts of mouse genomic DNA are mixed with HeLa or CHO DNA, we estimate that human transfectants have incorporated on the average considerably less than 0.02% of the mouse genome. The CHO-9-related cells contained in the order of 0.2% of the mouse complement per cell.

To study the average integrity of the DNA fragments incorporated in the

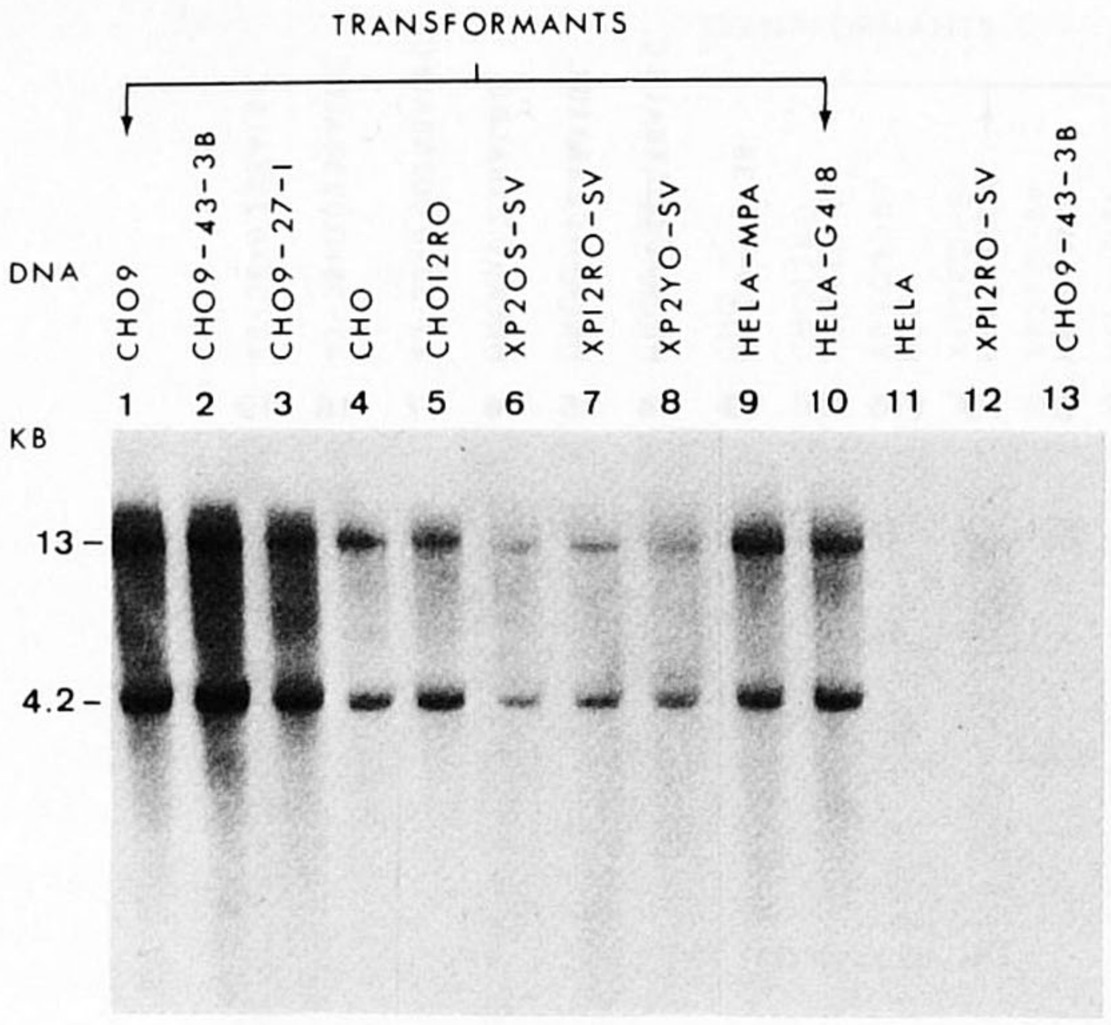

Fig. 4. Analysis of the average integrity of transfected DNA sequences incorporated in the genome of CHO, and human transformants. DNA (10 μg/lane) of the transformed cell populations indicated was digested with Bgl II and Sal I, generating from each copy of cosmid cos 43-34 a 13 kb Bgl–Bgl and a 4.2 kb Bgl–Sal fragment (see fig. 1) both containing pBR sequences. These fragments were visualized by hybridization with a ^{32}P-labelled pBR322 probe (see caption to fig. 2 for further details).

genome, DNA from the pooled transformants of each cell line was digested with Sal I and subsequently with Bgl II, yielding two fragments of cos 43-34 (4.2 and 13 kb) which can be identified with a pBR322 probe (fig. 1). As these two fragments were present in the transfected DNA in equimolar amounts, approximately the same intensity of hybridization is expected if no preferential degradation of large DNA has occurred. As shown in fig. 4, the intensity of the large 13 kb fragment is less than that of the 4 kb fragment, indicating that a smaller proportion of the larger fragment has remained intact during the transfection process. The broken fragments contribute to the background smear observed in the lanes. (This also provides an explanation for the background hybridization visible in fig. 2.) One cell line (XP2YO-SV, fig. 2, *8*) displays a very low 'survival' of the large fragments. The ratio of intensities of the large and small fragment in the other lanes shows relatively small variations. This indicates that the activity which causes rearrangement or degradation of exogenous DNA molecules does not vary dramatically between most of the cell lines tested. Hind III fragments of the cosmid of a size of 27 kb (see fig. 1) were also detected in all cells (except XP2YO-SV), the proportion of intact fragments of this size was again considerably smaller than that of the 13-kb fragment in fig. 4 (data not shown).

DISCUSSION

The work presented here shows that there are marked cell line-specific differences in the average amount of exogenous DNA stably inserted into the genome of transfected cells. The CHO cells (and particularly those of CHO-9 origin) incorporate much more DNA than the human cells in our panel. Southern blot analysis of individual clones of the various XP, HeLa and CHO transformants

agreed well with these findings, although we have observed significant variation in the quantity of exogenous sequences between different transformants of the same cell line (unpublished observations). Preliminary findings with one XP-G and one Fanconi's anemia SV40-transformed fibroblast line and a teratocarcinoma cell line (P3) were comparable to the results reported here for the XP cells. This indicates that we have not accidentally selected human cell lines that behave abnormally in this respect.

The limited data in the literature on individual transformants of human cells indicate that low copy numbers or small amounts of transfected sequences are frequently found (e.g. [24–26]). Rodent cells seem to be more suitable with respect to this parameter, although variation occurs between different rodent cell lines. Two V79 and one BHK cell line, subjected to the same set of tests as described here, appeared to behave in roughly the same way as HeLa cells (unpublished observations). This shows that some hamster cell lines can be inefficient in this respect as well.

Assuming that the uptake of dominant marker DNA and *ERCC-1* is representative of the incorporation of the other co-transfected DNA, a maximum estimate can be made of the average amount of exogenous sequences integrated in the XP and CHO cells. The XP-transformants harbor on the average 2–3 copies of the dominant markers (fig. 2). Consequently, they have inserted 2–3 cosmid molecules and three times as much mouse carrier DNA, amounting to about 500 kb in total. The same calculation for the CHO-9 group of transformants with about 100 copies of dominant marker genes leads to an estimated size of incorporated exogenous DNA of around 20000 kb. Intermediate values are obtained for the two CHO lines and HeLa. Since the transformants were only selected for the incorporation of a dominant marker gene, it is possible that the calculated values for the total amount of non-selected co-inserted DNA are overestimated. The finding that the non-selected dominant marker in the case of the human cells is also present and expressed in 60% up to over 90% of the transformants, together with the hybridization results in fig. 2, argues against a large difference between the uptake of the selected and non-selected DNA. Furthermore, the calculated amounts of incorporated DNA are in reasonable agreement with the average amount of integrated mouse carrier DNA as deduced from the data in fig. 3. The CHO-9 group shows a hybridization intensity close to the 0.2% titration in fig. 3, *19* which is equivalent to about 12000 kb mouse carrier DNA. The amount of mouse DNA in XP cells is a factor 30–100 lower (i.e. equivalent to 400–120 kb). The mean size of the integrated DNA we calculate for CHO-9, is in the same order of magnitude or slightly higher than that reported for Ltk^- [27].

Concerning the integrity of transfected DNA, no large differences in the extent of fragmentation or rearrangement of exogenous sequences were observed between the cell lines in our panel. An exception is the XP2YO-SV (XP-F) which, in all hybridizations, consistently exhibited the lowest signal and it has also the lowest transformation efficiency for dominant marker genes. Wake and co-

workers [28] have noted the occurrence of ds breaks in transfected DNA molecules on the average every 5–15 kb in monkey CV-1 cells. This is not so very different from the findings presented in fig. 4. The fact that cells with a low uptake of foreign DNA do not exhibit a higher extent of fragmentation suggests that this low uptake is not caused by increased breakage of the introduced DNA.

If human cells take up on the average 200–300 kb (as demonstrated here for XP cells) over 10^4 transformants will be required to cover the haploid human genome only once. With a transfection frequency of approx. 10^{-4}–10^{-5} this involves more than 10^8–10^9 cells. The occurrence of ds breaks on the average every 5–15 kb further reduces the number of intact gene copies, which could easily raise the number of cells to be transfected for the generation of one genomic transformant with a specific gene to over 10^9 cells. This is an order of magnitude at which experimentation becomes problematic. We think that these findings explain at least in part our inability (and that of others) to obtain repair-proficient transformants of the SV40-transformed XP fibroblasts using genomic DNA. The results of this study stress the importance of the recipient cell used for genomic DNA transfection experiments and may help to determine whether specific cells are suitable for these purposes.

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