Transformation of Human 143 tk- Cells with Plasmids Containing the Gene Encoding the Adenovirus DNA-binding Protein

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SUMMARY

Human cell lines that contain and express the gene encoding the adenovirus type 5 DNA-binding protein (Ad5 DBP) are very useful for the isolation of adenovirus mutants with an altered DBP. In order to obtain these cells, human 143 tk- cells were transfected, using the calcium phosphate technique, with plasmids containing the Ad5 DBP gene and the herpes simplex virus thymidine kinase (HSV tk) gene as a selectable marker. Characterization of several tk+ transformants revealed that these cells did contain the HSV tk gene, but in none of these cells could Ad5 DBP DNA sequences be detected. However, when 143 tk- cells were co-transfected with a plasmid containing the Ad5 DBP gene and another plasmid carrying early region E1, integration of the Ad5 DBP gene in chromosomal DNA could be detected. Integration of Ad5 DNA sequences was also observed when transfection was performed with plasmids containing the Ad5 DBP gene and the long terminal repeat of Moloney murine leukaemia virus. By employing a radioimmunoassay it could be shown that DBP-related proteins were synthesized in two of the cell lines containing the Ad5 DBP gene. Since both cell lines support the growth of the temperature-sensitive viral DBP mutant, H5ts125, at the non-permissive temperature, the DBP-related proteins expressed in these cells must be functional.

INTRODUCTION

DNA transfection offers the possibility of introducing new genetic information into mammalian cells. This technique is very useful for studying the expression of individual genes in detail. Wigler et al. (1977) and Bacchetti & Graham (1977) demonstrated that, by employing DNA transfection techniques, the herpes simplex virus thymidine kinase (HSV tk) gene can be stably integrated into the genome of cells with a defective tk gene (tk- cells). Transformants that express the HSV tk gene were isolated by growth in selective medium. It was further shown that non-selectable genes can also be integrated into the genome of tk- cells when these genes are linked to or co-transfected with the HSV tk gene.

We have focused our attention on the integration in human cells of a region of the adenovirus type 5 (Ad5) genome encoding the DNA-binding protein (DBP). This region, located between 61.5 and 75.1 map units (m.u.) on the Ad5 genome, is designated early region E2A and plays an important role in adenovirus DNA replication. Early in the productive infection cycle, transcription of this region is controlled by a promoter located at 75.1 m.u., whereas late in infection a promoter located at 72.0 m.u. is active. Early transcription of region E2A is stimulated by gene products encoded by region E1 (Berk et al., 1979; Shenk et al., 1979; Nevins, 1981; Montell et al., 1982; Rossini, 1983; Imperiale & Nevins, 1984). For our studies of integration of early region E2A in the genome of human cells we have constructed plasmids containing the Ad5 DBP gene as well as the HSV tk gene.

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In order to investigate whether stimulation of transcription influences the efficiency of stable integration we have performed transfection experiments in the absence and presence of early region E1. Further, we have used plasmids containing early region E2A and another transcription control element, the long terminal repeat (LTR) of Moloney murine leukaemia virus (Mo-MuLV). The LTRs of RNA tumour viruses contain highly conserved sequences thought to be essential for viral DNA synthesis (Schwartz et al., 1977) and for integration of the viral genome into the host chromosome (Hughes et al., 1978). LTRs also contain sequences involved in the initiation of transcription of the integrated viral genome and polyadenylation of mRNA. In addition, the LTRs appear to contain enhancer-like sequences (Blair et al., 1980; Huang et al., 1981; Jolly et al., 1983; Krieger & Botchan, 1983; Levinson et al., 1982; Wood et al., 1983). Recently, Luciw et al. (1983) demonstrated that the Rous sarcoma virus enhancer region is able to enhance the expression of the integrated HSV type 1 (HSV-1) tk genes in mouse L tk− cells, but has little effect on the efficiency of integration of the HSV-1 tk gene. Our experiments have revealed that the use of these elements facilitates the isolation of human cells containing and expressing the Ad5 DBP gene.

METHODS

Construction of plasmids. The construction of plasmids containing the LTR of Mo-MuLV and the genes of Ad5 DBP and HSV-1 tk is illustrated in Fig. 1. The LTR was isolated by digestion of the plasmid C6J Mol LTR2198 (a gift of Dr A. Berns, Nijmegen, The Netherlands) with HindIII and ClaI followed by agarose gel electrophoresis of the digest. The Ad5 HindIII-A fragment (50·1 to 72·8) was obtained by cleavage of whole Ad5 DNA with HindIII. This fragment contains the late promoter of the early region E2A at 72·0 m.u. and the complete coding sequences for Ad5 DBP (50·1 to 72·8 m.u.). Plasmid pAGO containing the HSV-1 tk gene was provided by Dr A. J. van der Eb (Leiden, The Netherlands) and was originally described by Colombé-Garapin et al. (1979). Plasmid pH2tk, a cloning derivative of pBR322 containing the HSV type 2 (HSV-2) tk gene, was a gift of Dr J. Walboomers (Amsterdam, The Netherlands). Plasmid pXho-C containing the region 0 to 15 m.u. of Ad5 DNA (early region E1) was obtained from Dr A. J. van der Eb.

Two plasmids, pAdLTR1 and pAdLTR2, containing the Moloney LTR, the Ad5 HindIII-A fragment and the HSV-1 tk gene were constructed. Both plasmids have a size of 15 kb and differ only in the relative orientation of the Ad5 HindIII-A fragment. In addition, plasmid pAd2, which resembles pAdLTR2 but does not contain the LTR sequences, was constructed. Further, plasmid pBZ1 was obtained by insertion of the Ad5 HindIII-A fragment into the HindIII site of pH2tk (Fig. 2).

The general procedure for molecular cloning of the constructs has been described by Maniatis et al. (1982). DNA fragments were fractionated by agarose gel electrophoresis and eluted according to the electro-elution procedure described by Maniatis et al. (1982). Escherichia coli strain C600 was used for cloning and propagation of the plasmids described in this study. For preparative purposes, large cultures of bacteria were grown, harvested and treated with lysozyme and Triton X-100 and used for purification of plasmid DNA. All cloning experiments were performed under defined conditions for recombinant DNA manipulation.

Transfection of human 143 tk− cells and characterization of tk+ transformants. Human 143 tk− cells obtained by selection with bromodeoxyuridine and the virus-transformed human cell line R970-5 (Rhim et al., 1979) were used for transfection employing the calcium phosphate precipitation technique as described by Graham & Van der Eb (1973) with modifications as proposed by Chu & Sharp (1981). Transfections were carried out in the absence of carrier DNA to stimulate integration of foreign DNA (Huttner et al., 1981; Scangos et al., 1981). Each individual tk+ colony was grown to a density of approximately 109 cells. High molecular weight cellular DNA was isolated by conventional procedures (Wahl et al., 1979) and, after cleavage with restriction enzymes, blotted on nitrocellulose filters by the method of Southern (1975). Hybridization of the filters with nick-translated probes was performed as described by Wahl et al. (1979).

Radioimmunoinhibition assay for DBP. DBP, a gift of Dr P. C. van der Vliet (Utrecht, The Netherlands) was labelled in vitro with 125I as described by Bolton & Hunter (1973) with minor modifications. One µg DBP was added to the vacuum-dried Bolton–Hunter reagent (Amersham) and incubated overnight at 0 °C in a volume of 25 µl (10 mM-Tris–HCl pH 8, 50 mM-NaCl, 0·1 mM-EDTA). Then phosphate-buffered saline (PBS) containing 1 mg/ml bovine serum albumin (BSA) (RIA grade, Sigma) was added to a final volume of 150 µl and the iodinated protein was exhaustively dialysed against PBS. A specific activity of 300000 c.p.m. 125I per µg protein was obtained. The IgG fraction of an antiserum against DBP was provided by Van der Vliet (Van der Vliet et al., 1977). Cellular extracts were prepared as described by Johansson et al. (1978). Cells were harvested, washed twice with PBS, suspended in PBS/TDS–PMSF (0·02 M-KH2PO4 pH 7·5, 0·1 M-NaCl, 0·5 % deoxycholate, 1 % Triton X-100, 0·1 % SDS, 1 mM-phenylmethylsulphonyl fluoride) to 107 cells/ml. This suspension was sonicated for 40 s and...
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Fig. 1. Construction scheme of pAdLTR1, pAdLTR2 and pAd2. The shaded region represents the HSV-1 tk gene, the black region represents a Clal/HindIII fragment containing the LTR of Mo-MuLV, and the open region indicates the Ad5 HindIII-A fragment containing the DBP-coding sequences. The letters C, H, K, S and B refer to cleavage sites for Clal, HindIII, KpnI, SalI and BamHI, respectively. The arrows indicate the direction of transcription of the genes.

Fig. 2. Structure of pBZ1, a plasmid containing sequences derived from pBR322 (circle), the HSV-2 tk gene and the Ad5 HindIII-A fragment. The shaded region represents the DBP gene. Numbers in parentheses refer to map positions on the Ad5 genome. H and K refer to HindIII and KpnI cleavage sites, respectively. The arrows indicate the direction of transcription.
incubated at 4°C for 1 h. A clear supernatant was obtained after centrifugation of the lysate (10000 \( g \), 15 min, 4°C).

To determine the optimal conditions for the radioimmunoinhibition assay, a fixed amount of the labelled antigen (\( ^{125} \)I-DBP) was incubated overnight at 4°C with different amounts of IgG in PBS/TDS, containing BSA (final concentration 1 mg/ml). After addition of Protein A-Sepharose the reaction mixture was incubated for 30 min at 0°C and centrifuged (15000 \( g \), 10 min, 4°C). The pellet was washed three times with PBS/TDS and the radioactivity present in the precipitate was determined. For the determination of the cross-reacting DBP in cell lines \( H25 \) and \( H65 \), the amount of antiserum that precipitated 30 to 50% of the precipitable labelled antigens was used in the assay.

**Immunoprecipitation analysis of tk\(^+\) transformants containing DBP gene.** Adenovirus-infected HeLa cells and tk\(^+\) transformants were grown overnight in 6 ml MEM without methionine, supplemented with 5% dialysed calf serum containing 100 \( \mu \)Ci\(^{35} \)S methionine (1000 Ci/mmol, Amersham). Extracts were prepared and immunoprecipitation was performed as described. The immunoprecipitated proteins were analysed on a 10% polyacrylamide gel.

**Plaque assay.** Plaque titrations were performed as described by Williams (1970) at 32°C and 39.5°C with 293 cells, a human embryonic kidney cell line morphologically transformed with E1A and E1B genes of Ad5 (Graham et al., 1977; Aiello et al., 1979).

**RESULTS**

**Transfection of human 143 tk\(^-\) cells with plasmids carrying the HSV-1 tk and the Ad5 DBP genes**

Human 143 \( tk^- \) cells were transfected with a variety of plasmids using the calcium phosphate technique as described in Methods. Since Huttner et al. (1981) have shown that omission of carrier DNA enhances the efficiency of stable integration of foreign DNA into the human genome, transfection was performed in the absence of carrier DNA.

The plasmids used were pAGO and a number of pAGO derivatives (pAGO-LTR, pAd2, pAdLTR1 and pAdLTR2). All derivatives carry the HSV-1 tk gene and all, except pAd2, contain the LTR of Mo-MuLV. pAdLTR1 and pAdLTR2 contain in addition the gene encoding Ad5 DBP in both orientations. pAd2 contains the Ad5 DBP gene but this plasmid does not carry the Mo-MuLV LTR. For details see Fig. 1. The transformation frequency of human 143 \( tk^- \) cells into \( tk^+ \) cells by transfection with pAGO and pAGO-LTR was determined and yielded for both plasmids about the same efficiency, namely one or two \( tk^+ \) colonies/\( \mu \)g pAGO/\( 10^6 \) cells and two to four \( tk^+ \) colonies/\( \mu \)g pAGO-LTR/\( 10^6 \) cells, respectively, indicating that the LTR has no profound effect on integration and expression of the HSV tk gene.

Four independently isolated \( tk^+ \) transformants obtained by transfection of the human 143 \( tk^- \) cells with pAd2, four independently isolated \( tk^+ \) transformants obtained by transfection with pAdLTR1 and four \( tk^+ \) transformants isolated after transfection with pAdLTR2 were grown to mass culture in selective HAT medium. Cellular DNA was isolated and cleaved with the restriction endonuclease \( \text{HindIII} \), which recognizes two sites in the plasmids. After gel electrophoresis and blotting of the digested DNA, hybridization was performed with the \( ^{32} \)P-labelled Ad5 \( \text{HindIII-A} \) fragment. To our surprise it appeared that none of the four \( tk^+ \) transformants obtained by transfection with pAd2 contained Ad5 DNA sequences, despite the fact that Southern blotting revealed the presence of the HSV-1 tk gene (results not shown).

Absence of integration of Ad5 DNA sequences was also observed when 143 \( tk^- \) cells were transfected with a pAGO derivative containing the \( \text{BamHI}–\text{EcoRI} \) fragment (59-5 to 75-9 m.u.) of the Ad5 genome. In the latter case six \( tk^+ \) transformants were examined (results not shown). However, integrated Ad5 DNA sequences were present in six of the eight \( tk^+ \) transformants obtained by transfection with pAdLTR1 and pAdLTR2 (Fig. 3, lanes 2 to 6 representing five \( tk^+ \) transformants). The identical size of the Ad5 \( \text{HindIII-A} \)-containing cellular DNA fragments derived from the pAdLTR1- and pAdLTR2-transformed cells and the authentic Ad5 \( \text{HindIII-A} \) fragment (8 kb) indicates that the transformants contain an intact integrated copy of the Ad5 \( \text{HindIII-A} \) fragment. The intensity of the hybridizing bands of some cell lines suggests that in these cells multiple copies of the \( \text{HindIII-A} \) fragment have been integrated. Obviously, the presence of the Mo-MuLV LTR in the plasmids used for transfection facilitates the isolation of human cells containing the Ad5 DBP gene.
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In order to investigate the integration pattern of tk\(^+\) transformants in more detail, cellular DNA of transformant H25 was cleaved by different restriction endonucleases. After digestion, the cleaved DNA was blotted and hybridized with \(^{32}\)P-labelled DNA of the entire plasmid pAdLTR2 or with specific restriction enzyme fragments (a detailed map of the plasmid is shown in Fig. 1).

As can be deduced from the blots (Fig. 4, lanes 1 and 4) one main band co-migrated with the linearized plasmid after digestion with \(ClaI\) when the Ad5 HindIII-A fragment and pAdLTR2 were used as probes, respectively. In addition, two other bands of approximately 6 to 8 kb are visible after hybridization with pAdLTR2 (Fig. 4, lane 1). Digestion of the cellular DNA with \(SalI\), which also cleaves pAdLTR2 once only (see Fig. 1), yielded one main band which co-migrated with the linearized plasmid (15 kb) and two additional bands of approximately 6 to 8 kb when hybridized with pBR322 and the tk gene as probe, respectively (Fig. 4, lanes 7 and 9). It is interesting to note that cleavage of cellular H25 DNA with \(ClaI\) and hybridization of the filter with Ad5 HindIII-A as a probe showed only one main hybridizing band co-migrating with the linearized plasmid (15 kb) (Fig. 4, lane 4). These results and the relative intensity of the bands suggest a tandem integration of five to ten copies of pAdLTR2 in a head-to-tail arrangement.

In a parallel experiment, human 143 tk\(^-\) cells were transfected with the plasmid pBZ1 and pBZ1 plus pXho-C, respectively. pBZ1 contains the HSV-2 tk gene and the HindIII-A fragment of Ad5 DNA encoding DBP (Fig. 2). pXho-C is a pAT153 derivative containing the entire Ad5 early region E1 inserted in the SalI cleavage site. After 8 to 10 days of growth in selective medium individual colonies were picked and grown to mass culture. Six tk\(^+\) transformants tested were derived from transfection with pBZ1 alone and four tk\(^+\) transformants were obtained by co-transfection with pBZ1 and pXho-C. High molecular weight DNA was isolated and cleaved with the restriction enzyme HindIII. After gel electrophoresis, blotting of the digested DNA and hybridization of the blots was performed with \(^{32}\)P-labelled total Ad5 DNA and the HindIII-A
fragment, respectively. These experiments revealed that although Ad5 DNA sequences are linked to the HSV-2 tk gene in pBZ1, none of the six tk+ cells transformed by pBZ1 alone contained detectable amounts of Ad5 DNA, while all cells contained intact HSV-2 tk genes as shown by Southern blotting (results not shown). On the other hand, Ad5 DNA sequences could be detected in all four cell lines transformed by pBZ1 plus pXho-C.

DNA from one of the tk+ transformants (H65), obtained by co-transfection with pBZ1 and pXho-C, was analysed in more detail by restriction enzyme analysis. Hybridization of HindIII-digested H65 DNA with 32P-labelled Ad5 DNA revealed three major (bands, 1, 3 and 4) and two minor (bands 2 and 5) hybridizing bands (Fig. 5b). The three major bands were also detected when 32P-labelled Ad5 HindIII-A fragment was used as probe (Fig. 5e). Obviously, the two minor hybridizing bands represent pXho-C-derived sequences. These experiments indicate that the presence of early region E1 facilitates stable integration of other Ad5 DNA sequences in the genome of 143 tk- cells.

Detection of DBP in human 143 tk+ transformed cell lines

The presence of Ad5 DBP sequences in 143 tk+ transformants obtained by cell lines transfected with pAdLTR1 and pAdLTR2 or co-transfection with pBZ1 and pXho-C raised the question of whether the integrated Ad5 DNA sequences are transcribed and expressed in these cell lines. Therefore, two human 143 tk+ transformants, H25 and H65, which were obtained by transfection with pAdLTR2 and pBZ1 plus pXho-C, respectively, were investigated for the presence of DBP. Both transformants contain DBP-coding sequences as demonstrated by Southern blotting analysis.

For the detection of DBP-related proteins in cell lines H65 and H25, samples of whole cell extracts of both cell lines were incubated with 40 ng 125I-labelled DBP and a limited amount (1 μl) of the IgG fraction of an anti-DBP serum. Incubation was performed at 4 °C in 500 μl PBS/TDS containing BSA (RIA grade, Sigma) (1 mg/ml). After incubation of the samples,
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Fig. 5. Integration pattern of Ad5 DNA in the genome of human 143 tk+ transformant H65, as revealed after cleavage of the cellular DNA with HindIII restriction endonuclease, blotting and hybridization with 32P-labelled Ad5 DNA (a to d) or 32P-labelled Ad5 HindIII-A fragment (e to g). (a) Ad5-transformed rat kidney cell line 5RK20 (Visser et al., 1981); (b) H65 cellular DNA; (c) 143 tk- cellular DNA; (d) calf thymus DNA to which was added the equivalent of ten copies of Ad5 DNA per diploid cell genome; (e) cellular H65 DNA; (f, g) calf thymus DNA to which was added the equivalent of one and five copies of pBZ1 DNA, respectively, per diploid cell genome.

Fig. 6. Radioimmunoinhibition assay of DBP in the transformants H25 (△) and H65 (□), in Ad5-infected HeLa cells (■) and in human 143 tk- cells (●). Various amounts of extract were incubated with 40 ng 125I-labelled DBP (sp. act. 3 × 10⁵ c.p.m./μg) and a limited amount (1 μl) of the IgG fraction of the anti-72K protein serum. y represents the relative fraction of labelled antigen precipitated. The amount of DBP present in cell line H25 and H65 was approximately 12 ng/10⁶ cells and 5.5 ng/10⁶ cells, respectively. The Ad5-infected HeLa cells yielded 100 ng DBP/10⁶ cells early in infection.
Protein A-Sepharose was added as described in Methods. The amount of DBP cross-reacting material was calculated according to the method of Murphy (1967). In both cell lines DBP was expressed in detectable amounts (Fig. 6); cell line H25 contained 12 ng DBP/10^6 cells while cell line H65 contained 5.5 ng DBP/10^6 cells. These amounts of DBP cross-reacting material represent 5 to 12% of the amount of DBP found in Ad5-infected HeLa cells early in infection (100 ng/10^6 cells). In order to determine the size of the DBP cross-reacting material in cell lines H25 and H65, cells were labelled in vivo with [35S]methionine and subjected to immunoprecipitation analysis as described in Methods. The immunoprecipitates were subjected to PAGE. As shown in Fig. 7, cell line H25 contained a DBP-related protein of mol. wt. 70000 (70K) (Fig. 7b, 7c).
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growth of the temperature-sensitive DBP mutant H5ts125 in cell lines H25 and H65

In order to test whether the DBP-related protein present in cell lines H25 and H65 is functional and can be used to complement the temperature-sensitive DBP mutant H5ts125 (Van der Vliet et al., 1975), the following experiment was performed. Monolayer cultures of human 143 tk- cells and cell lines H25 and H65 were infected at 39.5 °C with 3 p.f.u./cell Ad5 wild-type virus (Ad5wt) or H5ts125, respectively. After 2 days the Ad5wt-infected 143 tk- cells showed a distinct cytopathic effect, while the morphology of H5ts125-infected 143 tk- cells could not be distinguished from that of uninfected cells. However, in both the H5ts125-infected H25 and H65 cells, a distinct cytopathic effect could be detected. This suggests that the DBP-related proteins present in the transformed cell lines H25 and H65 complement the temperature-sensitive defect of mutant H5ts125.

In order to compare the virus yields in H25 and H65 cells infected with Ad5wt and H5ts125, respectively, at 39.5 °C, the infected cells were freeze-thawed 3 days after infection and the cell lysates were titrated in 293 cells at 32 °C. These cells were used because virus titres measured by plaque assay on 293 cells were consistently 30- to 100-fold higher than the titres measured on 143 tk- cells (unpublished results). It appeared that the Ad5wt and H5ts125 titres in H25 were almost identical (0.9 × 10^5 and 0.8 × 10^5 p.f.u. per ml diluted cell lysate, respectively). Similar results were obtained with Ad5wt- and H5ts125-infected H65 cells (0.5 × 10^5 and 0.6 × 10^5 p.f.u./ml, respectively). To ensure that the virus produced at 39.5 °C by H5ts125-infected H25 and H65 cells was indeed H5ts125, the cell lysates were also titrated at the non-permissive temperature (39.5 °C). Under these conditions the virus titres measured by plaque assay on 293 cells appeared to be less than 10^2 p.f.u./ml. These results indicate that cell lines H25 and H65 support the growth of mutant H5ts125 at the non-permissive temperature with about the same efficiency as Ad5wt.

Discussion

The experiments described in Results demonstrate that transfection of human 143 tk- cells with plasmids containing the HSV tk gene linked to Ad5 DNA sequences leads very inefficiently to stable integration of Ad5 DNA in the human genome. This observation is in agreement with the results of Klessig et al. (1984a), who reported that when HeLa cells were transfected with plasmids containing the Ad5 DBP and the HSV-1 tk genes fewer than 3% of the tk+ transformants contained an intact DBP gene. In this respect, human cells differ from rat 2 tk- cells which very efficiently incorporate Ad5 DNA sequences when transfected with pBZ1 (six out of seven tk+ transformants contained the DBP gene, results not shown).

However, linkage in vitro of the Mo-MuLV LTR to the Ad5 DBP gene cloned in pAGO containing the HSV-1 tk gene increases the efficiency of stable integration of the DBP gene strikingly independent of the relative orientation of the LTR. Similar results were obtained when the DBP DNA sequences were co-transfected with a plasmid containing early region E1. Losses of input sequences accompanying the integration and stabilization events are probably responsible for the observed low efficiency of stable integration of Ad5 sequences after transfection with plasmids carrying both HSV-1 tk and Ad5 DNA sequences. The extent to which input sequences are lost is apparently influenced by the presence of the Mo-MuLV LTR or Ad5 region E1. How the LTR or the region E1 gene products exert such an effect is difficult to explain. Since transfection with either pAGO or pAGO-LTR leads to similar frequencies of tk+ transformation, it is unlikely that the presence of the LTR directly influences the processes of uptake and insertion. It is possible that the presence of LTR enhances the stability and/or copy number of the transfected plasmids, thereby increasing the probability of stable integration of the Ad5 DNA sequences linked to the HSV-1 tk gene. The presence of multiple copies of pAdLTR2 in the H25 cell line might be significant in this respect. Another possibility is that the Mo-MuLV LTR affects the level of transcription of the Ad5 DBP gene and that this stimulation of transcription in a not yet understood manner leads to enhanced stable integration of this gene. In this respect it is interesting that Chang et al. (1980), Copeland et al. (1981), Kriegler &
Botchan (1983) and Luciw et al. (1983) have demonstrated that LTRs are not directly involved in the recombination process but that these repeats affect the level of expression of transfected genes.

The stimulating effect of Ad5 region E1 on stable integration of the DBP gene is equally difficult to explain. Products of early region E1A are known to stimulate expression of early adenovirus genes during the productive infection cycle. This raises the question of whether the presence of region E1A enhances the stable integration of the DBP gene by stimulation of its expression. It should be pointed out that the stimulation of expression of the DBP gene by E1A gene products is only observed for the early promoter of DBP gene located at 75-1 m.u. and not for the late promoter at 72-0 m.u. Since in pBZ1 only the late promoter at 72-0 m.u. is present, it is unlikely that the increased efficiency of integration of the Ad5 DBP gene is due to the specific transcription-stimulating effect of region E1A gene products on the DBP gene. On the other hand, it is also known that region E1A proteins may have a general stimulating effect on transcription of exogenously introduced genes (Treisman et al., 1983; Green et al., 1983; Gaynor et al., 1984; Svensson & Akusjärvi, 1984; Kingston et al., 1984). Kingston et al. (1984) have proposed that region E1A proteins cause a general change of the transcription apparatus resulting in the stimulation of promoters of genes, introduced into cells by micro-injection. On the other hand, Alwine (1985) reported that apparently non-specific effects of region E1A on transcription of plasmids turned out to be artefacts due to differential replication and stability of different plasmids in different cell lines. If this also holds for the plasmids and cell lines used in this study, a stimulating effect of region E1 products on stable integration by enhancement of transcription is not very likely. In that case, as mentioned for LTRs, region E1 products might enhance the stability and/or copy number of the transfected plasmids resulting in an increased probability of stable integration of Ad5 DNA sequences linked to the HSV-2 tk genes.

In conclusion, we observed that stable integration of non-selectable exogenously introduced DNA into the human genome is strongly enhanced by the presence of a retrovirus LTR or Ad5 region E1, but the precise mechanism of stimulation is still unknown.

Two cell lines (H25 and H65) which contain the gene for Ad5 DBP were characterized with respect to expression of this gene. Employing a radioimmunoinhibition assay it could be shown that DBP-related proteins are synthesized in both cell lines. When a [35S]methionine-labelled cell extract of cell line H25 was immunoprecipitated with anti-DBP serum a detectable amount of protein with a mol. wt. of 70K was immunoprecipitated. This protein has a lower mol. wt. than authentic DBP (72K). This lower mol. wt. might indicate that the 70K protein is a truncated translation product. However, it is also possible that the 70K protein is underphosphorylated when compared with the DBP isolated from productively infected HeLa cells, leading to a lower apparent molecular weight. Further, it could be shown that the DBP synthesized in H25 cells is functional, because these cells are able to support growth of a mutant with a defect in the DBP gene (H5ts125). It is interesting that even cell line H65 supports the growth of the H5ts125 mutant, despite no immunoprecipitable protein being detected in these cells. Therefore, the H25 and H65 lines are very useful for the isolation and characterization of new mutants with a defect in the DBP gene.

Recently, Klessig et al. (1984b) showed stable integration and controlled expression of the DBP gene in HeLa cells under the influence of the LTR of mouse mammary tumour virus whose activity is induced by glucocorticoid hormones (Ringold et al., 1975). The E. coli gene coding for xanthine–phosphoribosyltransferase guanine was used as dominant selectable marker that allows cells, which contain and express this gene, to grow in the presence of mycophenolic acid (Mulligan & Berg, 1981). The DBP gene was expressed at a low level in HeLa cells (5 to 15% of the level seen at peak time in infected HeLa cells) when dexamethasone was added for a short period of time. This low level seemed to be sufficient to support virus growth for 3 to 5 days after infection. However, after prolonged growth of the cells in the presence of dexamethasone, these low levels of DBP were toxic for the cells. These results are in contrast with our observations. We have demonstrated that with the same level of DBP, cell lines H25 and H65 are able to express constitutively DBP-related proteins for at least 6 months (results not shown). Possibly, HeLa and 143 tk cells differ in their sensitivity to DBP and DBP-related proteins.
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