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Adenovirus late sequences linked to herpes simplex virus thymidine kinase may be introduced into eukaryotic cells and transcribed

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ABSTRACT

LTK$^-$ cells have been transformed to the TK$^+$ phenotype by treatment with size-defined concatamers of HSV-1 TK DNA and Ad2 Bam H1 C fragment (42.0 - 59.5 map units). All TK$^+$ transformants contained Ad2 DNA as well as HSV-1 TK sequences. In most cases several inserts of virus DNA were present, many in high copy numbers. Although no Ad2 transcription promoter was present in the transforming DNA, Ad2-specific sequences were detected in polyadenylated cytoplasmic RNA species from several cell lines.

INTRODUCTION

The thymidine kinase (TK) gene of herpes simplex virus (HSV) can be used to transform TK$^-$ cells to TK$^+$ phenotype. This can be achieved either by infection with virus inactivated by ultraviolet irradiation (1) or by transfection with fragments of HSV DNA (2,3,4). Other HSV genes situated close to TK in the virus genome and contained within the transforming DNA fragment may be incorporated into the recipient cell genome along with the TK gene and, under appropriate conditions, these non-selected genes may be expressed in the transformed cell (4,5). Subsequently this system of co-transformation with HSV TK has been exploited to introduce genes of diverse origins, for which no independent selection criteria are available, into TK$^-$ eukaryotic cells. The non-selected genes may either be linked physically to the TK gene by *in vitro* ligation or construction of a recombinant plasmid (6,7,8), or they may be mixed with the TK gene prior to transformation (9,10,11,12). There is convincing evidence that where suitable eukaryotic promoters are present such non-selected genes may be correctly transcribed and processed (6,12) and specific protein products produced (7,11,12).

Our interest is in the introduction of various genes of virus origin into cells. However most virus genes, with the possible exception of some early functions of nuclear DNA viruses, would lack conventional eukaryotic

promoter sequences and this deficiency might prevent their expression. The objective of this study was to examine the fate of integrated structural genes which are known to lack suitable promoters, and for this purpose we chose to use the Bam Hl C fragment (Bam C) of adenovirus type 2 (Ad2) DNA. This fragment (map units 42.0 - 59.5 on the adenovirus genome) spans part of the major late transcription unit of Ad2 and contains structural coding sequences for several late proteins. However it lacks the major Ad2 late promoter (situated near 16.5 map units (13)) and also the three leader sequences which comprise the 5'-terminal untranslated region of late Ad2 mRNAs (14,15,16).

We report the construction of DNA concatamers containing the Ad2 Bam C fragment and the TK-containing Bam Hl fragment of HSV type 1 DNA (HSV Bam TK) and the use of size-defined hybrid multimers to transform mouse LTK^- cells to TK^+ phenotype. The virus DNA content of the cells has been analysed and we show evidence for transcription of both selected and non-selected sequences.

MATERIALS AND METHODS

Cells and viruses

LTK^- cells and Hep-2 cells, used for propagation of Ad2, were grown in Glasgow-modified Eagle's medium supplemented with 10% tryptose phosphate broth and 10% new-born calf serum. All LTK^+ transformants were selected and maintained by supplementing the above medium with thymidine, adenosine, guanosine, glycine and methotrexate (1). Ad2 virus was obtained from Dr. P. Gallimore (Department of Cancer Studies, Birmingham University).

Virus DNAs

Ad2 DNA was prepared from purified virions according to the method of Pettersson & Sambrook (17). The source of the HSV-1 TK gene was the plasmid xl (p-xl) described by Enquist *et al.* (18) which was generously provided by Dr. W. Summers. This contains the 3.5 Kb HSV-1 TK-containing Bam Hl fragment cloned into the Bam Hl site of pBR322. p-xl was grown in MRC1, a disabled strain of *E. coli* K12 (Dr. S. Brenner, Medical Research Council Laboratory of Molecular Biology, Cambridge, England) and was purified according to the method of Miller (19). Restriction map data for the HSV sequence in p-xl was taken from reference 18 and for Ad2 from Appendix D of reference 20.

Enzyme reactions

Restriction endonucleases and T4 DNA ligase were supplied by Bethesda Research Laboratories, Inc. and incubations were carried out using conditions

recommended by the manufacturers.

Agarose gel electrophoresis

DNA fragments were separated by overnight electrophoresis at 125V in horizontal agarose gels using Tris/acetate buffer (0.04 M Tris, 0.008 M Na acetate, 0.0004 M Na_2 EDTA, pH 7.85).

Purification of DNA fragments

Bands were excised from ethidium bromide stained agarose gels and the DNA recovered by binding to glass powder (21).

Transformation

LTK^- cell cultures were inoculated with DNA using the calcium technique (22) as described by Minson *et al.* (4) except that sheared LTK^- cell DNA was used as carrier.

Cell DNA preparation

Cells were washed once in PBS and resuspended in NET buffer (0.1 M NaCl, 0.01 M Tris, 1 mM EDTA, pH 7.5). SDS was added to 1% and the suspension heated at 60°, 5' and cooled. Following digestion with pronase (pre-digested 2 hr, 37°) at 37°, 5 hrs, the sample was extracted three times with phenol: chloroform (1:1, NET-saturated). 0.1 M NaCl was added to the final aqueous phase followed by 2.5 vols ethanol. DNA was collected by spooling and was re-dissolved, re-precipitated and spooled a further 3 times. Samples were finally dissolved at 1 mg/ml in 0.01 M Tris, pH 7.9.

DNA blots

Restriction digests of transformant cell DNAs were electrophoresed in 1% agarose gels and transferred to nitrocellulose filter sheets (Schleicher & Schüll BA85) (23). Pre-hybridisation, hybridisation and washing of the blots were esentially as previously described (24) except that sodium phosphate (pH 6.5) was added to 1 mM during pre-hybridisation and hybridisation steps and to 10 mM in the first wash buffer. In addition hybridisation and the first two sets of washes were at 65°; the last two low salt washes were at 55°. ^{32}P-labelled probes of specific activities 2-4 x 10^8 c.p.m./µg were prepared by nick translation (25) of purified DNA fragments using (α-^{32}P) dCTP (specific activity 2,000-3,000 Ci/mmol; The Radiochemical Centre, Amersham, England).

RNA preparation

Poly(A)-containing cytoplasmic RNA was prepared from transformants as previously described (26) except that two cycles of oligo(dT)-cellulose

chromatography were carried out to improve the purity of the poly(A)-containing fraction.

RNA blots

RNA preparations were denatured by heating to 60° for 5 min in 50% (v/v) formamide, 2 M formaldehyde and 1 x MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0). After addition of bromophenol blue and glycerol, samples were electrophoresed at 50 mA for 2 hrs on vertical 1.5% agarose gels containing 1 x MOPS buffer and 2 M formaldehyde. Gels were soaked 1 hr in 20 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M Na citrate) and RNA transferred to nitrocellulose filters by blotting (27). After baking for 2 hrs at 80°C in a vacuum oven, RNA blots were pre-hybridised overnight at 42° in 50% (v/v) formamide, 5 x SSC, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.1% SDS, 50 mM sodium phosphate pH 6.5, and 200 µg/ml denatured calf thymus DNA. Hybridisation buffer contained 50% formamide, 5 x SSC, 0.02% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin, 20 mM sodium phosphate pH 6.5, 0.1% SDS, 100 µg/ml denatured calf thymus DNA and 10% dextran sulphate. ^{32}P-labelled probes were prepared as described above for DNA blots. Hybridisation was overnight at 42° in a shaking waterbath, following which blots were washed with three changes of 2 x SSC, 0.1% SDS for 5 min each at room temperature, then with three changes of 0.1 x SSC, 0.1% SDS for 15 min each at 50° (27). The blots were dried and autoradiographed at -70° using intensifying screens. Removal of hybridised probe was accomplished by two washes of 5 min each in H_2O at 100° (27). Pre-hybridisation and hybridisation steps were then repeated with a second probe.

Preparation of ^{35}S-methionine labelled cell extracts and immunoprecipitation

Cell monolayers were incubated 8 hrs with 125 µCi/ml ^{35}S-methionine (500 Ci/mmol, The Radiochemical Centre, Amersham, England) in medium lacking unlabelled methionine. Cell extract preparation and immunoprecipitations using Sepharose-linked Staphylococcal protein A adsorption were as described by Inglis & Mahy (28). Rabbit antiserum to Ad2 virus was a kind gift from Dr. V. Mautner (M.R.C. Virology Unit, Glasgow). Polypeptides were separated by electrophoresis (35 mA, 4 hrs) on a 15% polyacrylamide slab gel overlaid with a 3% stacking gel (29). Labelled polypeptides were detected by autoradiography of dried gels.

RESULTS

Preparation of concatamers for transformation

The 3.5 Kb Bam Hl fragment of HSV-l DNA which encodes the TK gene (HSV Bam TK) was prepared by Bam Hl digestion of the pBR322 recombinant plasmid p-xl (18) followed by agarose gel electrophoresis and recovery of the appropriate fragment. The Ad2 Bam C fragment was similarly isolated from Bam Hl digested Ad2 DNA. Approximately equal numbers of these fragments were mixed and incubated with T4 DNA ligase. The resulting products were separated by electrophoresis on an agarose gel and compared with the products obtained by self-ligating each of the two fragments independently (Figure 1A). Bands appearing uniquely among the products of the mixed ligation reaction (track 3) and which therefore contained both types of fragment were excised and the

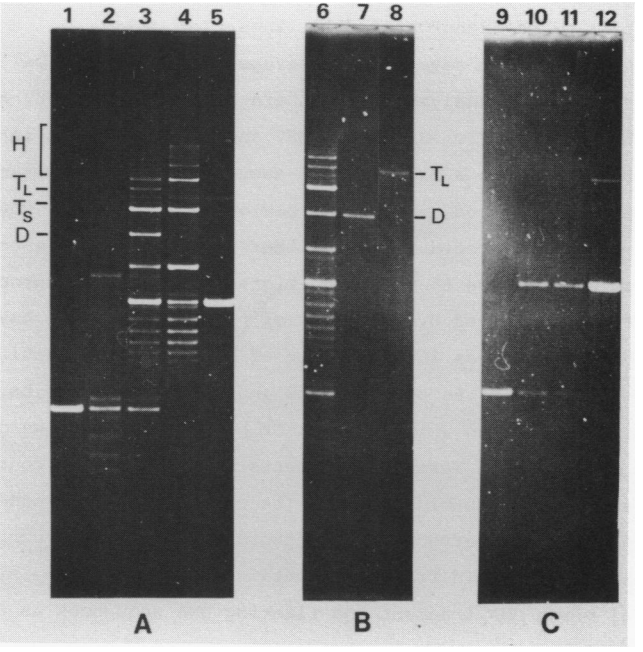

Figure 1. Preparation of hybrid concatamers.
A. Ligation products: 1. HSV Bam TK, 2. HSV Bam TK + ligase, 3. HSV Bam TK + Ad2 Bam C + ligase, 4. Ad2 Bam C + ligase, 5. Ad2 Bam C.
B. Recovery of individual bands: 6. HSV Bam TK + Ad2 Bam C + ligase, 7. recovered band D, 8. recovered band T_L.
C. Redigestion of recovered bands: 9. HSV Bam TK, 10. recovered band D + Bam Hl, 11. recovered band T_L + Bam Hl, 12. Ad2 Bam C.
DNAs were separated by agarose gel electrophoresis, stained with ethidium bromide and visualised over ultraviolet light.

DNAs recovered separately (Figure 1B). On the basis of gel migration distances these DNAs were designated as dimers (D), or trimers (T) containing either two TK fragments and one Ad2 Bam C fragment (T_S) or two Ad2 Bam C fragments and one TK fragment (T_L). Redigestion with Bam Hl of D and T_L species supported these assignments (Figure 1C). In addition the unresolved material from the high molecular weight region of the gel (H), representing multimers with four or more components, was recovered.

These recovered ligation products were used to transform LTK$^-$ cells to TK$^+$ phenotype using the calcium phosphate technique as described previously (4). Individual colonies were picked and expanded into cell lines. Transformants derived using the dimer DNA were designated Ad D lines, those derived using the trimer DNAs, Ad T_S and Ad T_L, and those derived using high molecular weight DNA, Ad H. A control cell line transformed with the HSV Bam TK fragment only was designated Ad 0.

Virus DNA content of co-transformed cell lines

DNA preparations from several low passage co-transformed cell lines were digested with Bam Hl and analysed by Southern blotting using HSV Bam TK and Ad2 Bam C probes (Figure 2). Since the HSV and Ad2 components in transforming DNA had been joined at a Bam Hl site, assuming that no rearrangements of virus sequences had occurred, any joined HSV and Ad2 fragments would be separated by this digestion procedure. If either fragment were present in its entirety it would give rise to a band co-migrating with the appropriate control Bam Hl fragment derived by digestion of p-xl or Ad2 DNA. Any such fragment must be derived from an internal site of an integrated multimer in order to have retained Bam Hl sites at both ends and would therefore be expected to occur only in cell lines in which trimers or higher molecular weight concatamers had been integrated. Terminal fragments from integrated concatamers would not be expected to co-migrate with the control Bam Hl fragments as Bam Hl sites would not be conserved at the integration points and consequently Bam Hl digestion would yield fragments containing both the remaining portion of the terminal virus DNA fragment and flanking DNA sequences as far as the nearest Bam Hl site.

Most co-transformants had clearly acquired multiple copies of both Ad2 and HSV sequences. Complete HSV Bam TK fragments appeared to be present in Ad T_S1, Ad T_L1, Ad H2, Ad H3 and probably Ad H1 (Figure 2A). The presence of bands detected by the Ad2 Bam C probe which migrated similarly to the control Ad2 Bam C fragment suggested that complete Ad2 Bam C fragments were also present in Ad T_S1, Ad H1, Ad H2 and Ad H3 (Figure 2B). As expected no com-

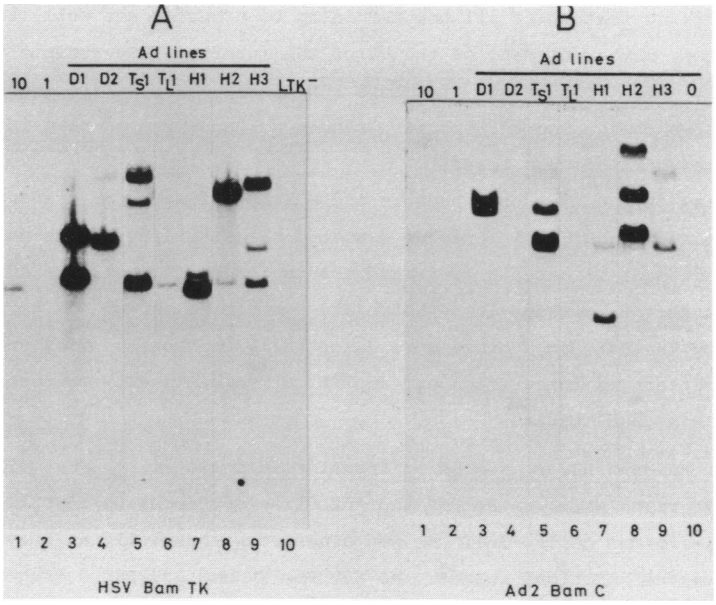

Figure 2. Southern blots of Bam H1 digested DNA from co-transformant cell lines. Blots were hybridised with (A) HSV Bam TK and (B) Ad2 Bam C probes and autoradiographed. Positive controls (tracks 1 & 2 of A & B) contained 10^7 and 10^6 copies of p-xl (Blot A) or Ad2 DNA (Blot B) mixed with LTK⁻ DNA before Bam H1 digestion, and are approximately equivalent to 10 and 1 copies/cell. All tracks were loaded with 15 μg of DNA.

plete fragments of either type were detected in the dimer transformants Ad D1 and Ad D2. All cell lines also yielded fragments containing virus DNA sequences which did not co-migrate with the relevant intact fragment and which therefore span integration sites and contain an incomplete virus DNA fragment (from which sequences at one or both ends have been lost) plus flanking DNA sequences. With the single exception of Ad T_L1, all co-transformants yielded more than two such terminal fragments suggesting that there were two or more independent inserts of transforming DNA in these cell lines. Ad T_L1 however yielded only two non-comigrating fragments containing virus DNA, both detected by the Ad2 Bam C probe (visible on longer exposure of the autoradiograph shown in Figure 2B). This cell line also appeared to contain the complete HSV Bam TK fragment (Figure 2A). All these fragments were represented at less than 10 copies/cell (by comparison with the reconstruction experiments (track 1 of each blot) which were equivalent to 10 copies/cell). Thus it seems likely that Ad T_L1 contains a single insertion of the trimer Ad2 Bam C - HSV Bam TK - Ad2 Bam C with some Ad2 sequences lost from each end.

A striking feature of all the remaining co-transformant cell lines was the high copy number of many of the virus DNA inserts. For example the cell line Ad Dl, which yielded two fragments containing HSV-specific sequences and a further two containing Ad2-specific sequences, appeared to have in excess of 50 copies/cell of each insert.

In view of the high copy numbers and multiple inserts of transforming DNA sequences in many cell lines we examined the stability of the non-selected Ad2 sequences in certain co-transformants (Ad Dl, Ad T_S1, and Ad H2). After a further 9 passages in methotrexate-containing medium the sequences detected by the Ad2 Bam C probe were essentially unchanged. This suggested that the pattern of integration and amplification of inserts was established early and was then stable.

Linkage of Ad2 and HSV sequences in transformed cells

To determine whether the HSV and Ad2 virus sequences were still linked in the transformed cells, cell DNA was digested with Bgl II, an enzyme which would leave the junctions intact. As HSV Bam TK and Ad2 Bam C fragments each contain a single Bgl II site (Figure 3) any inserted concatamer containing sequences extending out in both directions from the junction point of the two fragments, past the Bgl II site of each, would yield a junction fragment of predictable size which would hybridise to both HSV and Ad2 probes. There are four possible sizes for such intact junction fragments, namely 7.8 Kb, 5.7 Kb,

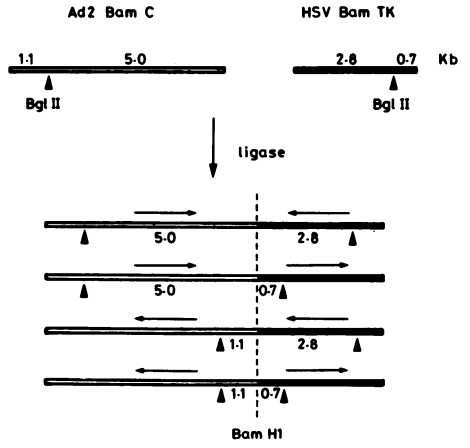

Figure 3. Diagram of junction fragments which can be generated by Bgl II digestion of Ad2 Bam C/HSV Bam TK concatamers. Vertical arrows show Bgl II sites; horizontal arrows show direction of transcription of Ad2 late genes and HSV TK gene.

3.9 Kb and 1.8 Kb, depending on the relative orientation of the two virus sequences (Figure 3). If either or both Bgl II sites were absent the size of the fragment generated would depend on the location of the nearest Bgl II site in the flanking DNA.

Southern blots of Bgl II digested co-transformant DNA were cut down the middle of each track; one half was hybridised with the HSV Bam TK probe and the other with Ad2 Bam C. The results (Figure 4) indicated that all cell lines examined contained at least one junction fragment which hybridised to both probes. Ad D2 contained a junction fragment of intermediate size indicating the loss of a Bgl II site, but in all other cases junction fragments of the predicted sizes were detected. Thus Ad D1 contained a 7.8 Kb junction fragment, Ad T_S1 contained 5.7, 3.9 and 1.8 Kb fragments, Ad H1 yielded 7.8 and 1.8 Kb fragments, Ad H2 5.7 Kb, and Ad H3 1.8 Kb junction fragments. All four possible relative orientations of the Ad2 and HSV TK DNA sequences therefore appeared to be represented in the cell lines studied. It is worth noting that in cases where the 5.7 Kb or 3.9 Kb junction fragment was detected the HSV and Ad2 fragments must be lined up so that TK transcription and Ad2 late protein transcription would be in the same direction.

Fragments which hybridised to only one of the two probes will include junctions between homologous fragments, terminal fragments spanning the

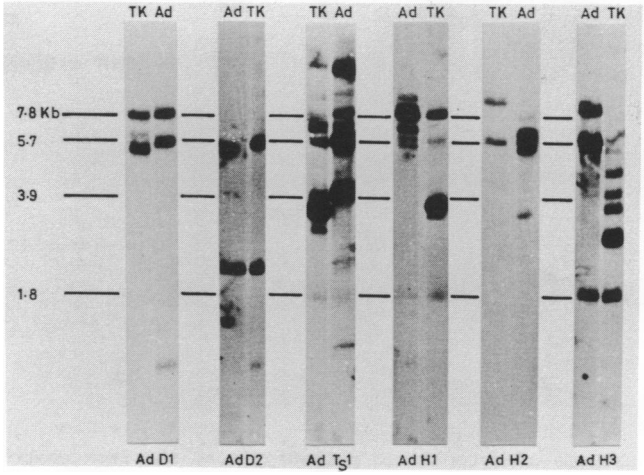

Figure 4. Southern blots of Bgl II digested DNA from co-transformant cell lines. Blots were cut down the middle of each track and one half of each hybridised with HSV Bam TK probe (TK) and the other with Ad2 Bam C probe (Ad) followed by autoradiography. Sizes were determined from the migration of co-electrophoresed unlabelled restriction fragments of Ad2 DNA.

integration site of one end of a concatamer into the flanking sequence, and any small inserts of either virus sequence.

Expression of integrated sequences

The data on virus DNA sequences in the co-transformants show that multiple copies of Ad2 sequences are present in most cell lines and that at least some of these sequences are joined to HSV TK sequences. Furthermore some cell lines (Ad T_S1, Ad H1, Ad H2 and Ad H3) appeared to contain intact Ad2 Bam C fragments (Figure 2). We next looked for expression of the non-selected Ad2 sequences at the level of cytoplasmic RNA. Polyadenylated cytoplasmic RNA preparations from co-transformant cell lines were separated by electrophoresis on agarose/formaldehyde gels and blotted onto nitrocellulose sheets. Figure 5 shows a single such blot hybridised firstly with Ad2 Bam C probe (Figure 5A), and secondly, after melting off the first probe, with the HSV Bam TK probe to detect TK transcripts (Figure 5B).

The TK probe revealed a major polyadenylated RNA species approximately

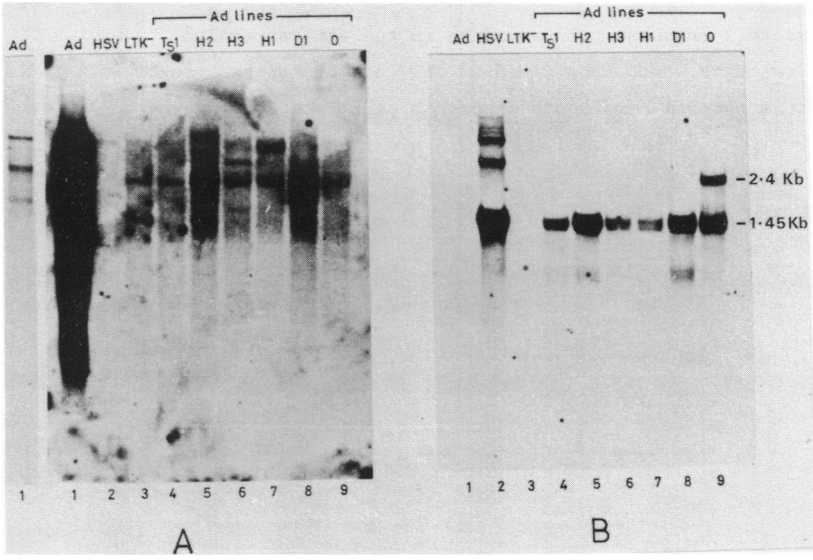

Figure 5. Analysis of cytoplasmic polyadenylated RNA from co-transformant cell lines. A single RNA blot was hybridised first with Ad2 Bam C probe (A) and secondly with HSV Bam TK probe (B) followed by autoradiography. Positive control tracks contained 0.4 µg of RNA from Ad2-infected Hep-2 cells 18 hr p.i. (1) and 0.4 µg of RNA from HSV-1 (strain F)-infected BHK cells 6 hr p.i. (2). All other tracks contained 4 µg of RNA. Autoradiograms were exposed for 21 days (A) and 5 days (B). A 5 hr exposure of track 1 from A is also shown.

1.45 Kb in size, present in all co-transformants and in Ad 0, which co-migrated with the major species detected in HSV-1-infected cells, and which was absent from untransformed LTK$^-$ cells. This species is believed to be the mRNA specifying the TK polypeptide, which must be produced in these cells in order to ensure survival in methotrexate-containing medium. A minor, faster-migrating RNA species of similar mobility in each case, was also detected in all transformed cell lines, but there appeared to be no corresponding species in the infected cell sample. Ad 0 contained an additional species, of higher molecular weight, which was not seen in any other sample.

The overall pattern obtained when the same blot was hybridised with the Ad2 Bam C probe was quite different (Figure 5A) and was complicated by the presence of a band which appeared in the LTK$^-$ and Ad 0 tracks, as well as in all co-transformants and which was presumed to be non-specific. Prominent cytoplasmic polyadenylated RNA species containing Ad2-specific sequences were however detected in three co-transformants, Ad H1, Ad H2 (which appeared to contain two distinct RNA species) and Ad H3. These RNAs were of different size in each cell line although all were apparently larger than 2.4 Kb. All these RNAs were within the approximate size range of RNAs detected by this probe in Ad2-infected cells. It is perhaps significant that the three lines in which transcripts of non-selected sequences were detected were all generated using high molecular weight concatamers. (The increased intensity of the non-specific band in Ad H2 and Ad D1 suggested the possibility that additional RNAs containing Ad2-specific sequences might have been masked by the non-specific hybridisation in this region of the blot). No RNA species were detected which contained both Ad2 and HSV sequences.

From this experiment it was therefore evident that the non-selected Ad2 sequences in these co-transformants were in several cases transcribed and the transcripts processed by polyadenylation and transported into the cytoplasm. This occurred in the absence of any Ad2 promoter. One processing signal known to be present in the Ad2 Bam C fragment however is the polyadenylation site at approximately 50 map units which represents the 3' co-terminus of the L2 family of late Ad2 mRNAs (30,31,32 and Figure 6). This site must be present in all cell lines containing intact Ad2 Bam C fragments and therefore in Ad H1, Ad H2 and Ad H3 and also in cell lines containing 7.8 or 5.7 Kb junction fragments (Figure 3) and therefore in Ad D1. Thus it was of interest to determine whether the RNA species containing Ad2-specific sequences had been polyadenylated at this site. Such RNAs would not contain sequences mapping to the right of 50 map units. To test this an RNA blot, prepared as before, was

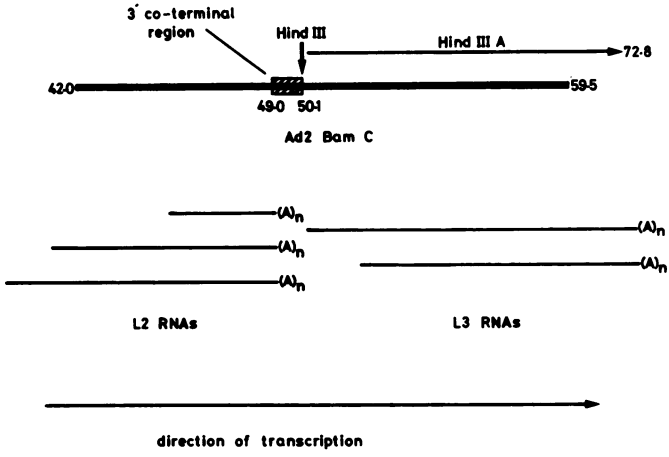

Figure 6. Diagram of Ad2 Bam C and the cytoplasmic RNAs encoded by this sequence in Ad2-infected cells, late in infection (30,31,32,35).

hybridised to an Ad2 Hind III A fragment probe (map units 50.1 - 72.8), specific for sequences downstream from the polyadenylation site (30). The results of this experiment (Figure 7) in fact showed that all but one of the RNA species detected previously (the single exception being that in Ad H3) contained downstream sequences and hybridised to this probe.

Since Ad2-specific sequences were transcribed into polyadenylated mRNA we examined the polypeptides in the co-transformant cells in an attempt to detect products antigenically related to Ad2 proteins. ^{35}S-methionine-labelled extracts of co-transformant cells were either analysed directly by polyacrylamide gel electrophoresis or after immunoprecipitation with anti-Ad2 antiserum (Figure 8). No virus-specific polypeptides were detected in any of the co-transformant cells tested.

DISCUSSION

Our objective in this study was to investigate the fate of non-selected DNA coding sequences incorporated into cells along with the TK gene of HSV. In these experiments we used coding sequences derived from the central portion of the adenovirus type 2 genome, specifically the Bam Hl C fragment (42.0 - 59.5 map units), which spans part of the major late transcription unit. This DNA fragment contains the structural coding sequences for several late Ad2 polypeptides (pVII, V, pVI, the C-terminal portion of III, and all

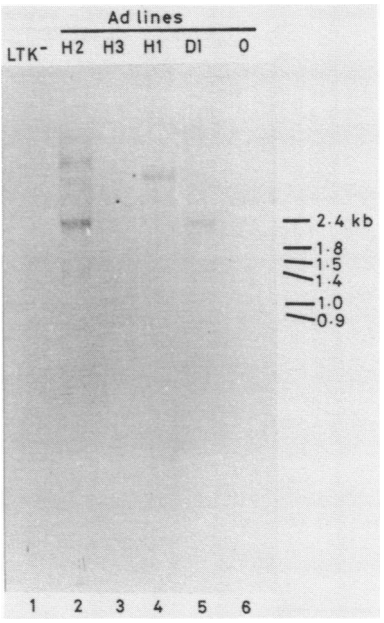

Figure 7. A blot of cytoplasmic polyadenylated RNAs (4 μg/track), hybridised with Ad2 Hind III A fragment probe and autoradiographed. Size markers were cDNAs to fowl plague virus mRNAs (provided by S.C. Inglis).

but the extreme C-terminus of II) (33,34) but not the late promoter (13) or any of the three leader sequences which, spliced together, become the untranslated 5' terminal sequences of late Ad2 mRNAs (14,15,16). However the Ad2 Bam C fragment does contain a polyadenylation site, utilised in the processing of the long primary transcript to produce the L2 family of late mRNAs (30,31,32) (specifying III, pVII & V (35)). Splicing acceptor sites for processing certain L2 and L3 mRNAs are also present. It was therefore of interest to examine whether this Ad2 sequence could be transcribed in transformed cells and if so whether any of the virus processing signals were recognised.

Mixed concatamers of Ad2 Bam C and HSV Bam TK fragments were generated by ligation and size-defined species, either dimers, trimers or mixed higher order multimers, were used to transform TK^- cells to TK^+ phenotype. DNA preparations from a number of these co-transformant cell lines were characterised by Southern blot analysis which revealed that in all cases tested conversion of cells to TK^+ phenotype by acquisition of HSV TK DNA was accompanied by acquisition of Ad2 DNA sequences. In most lines evidence obtained from Bgl II

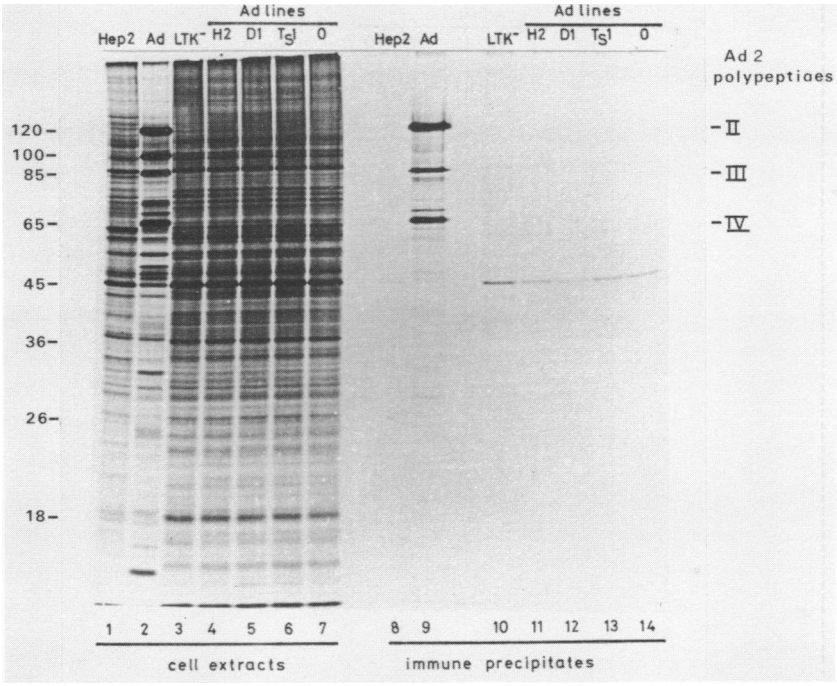

Figure 8. Polyacrylamide gel electrophoresis of ^{35}S-methionine labelled cell extracts before (tracks 1-7) and after (tracks 8-14) immunoprecipitation with anti-Ad2 antiserum. Ad2-infected and uninfected Hep-2 cells were labelled 19-24 hr p.i., other cells were labelled for 8 hrs. Tracks 1-7 were each loaded with approximately 3 x 10^5 TCA-precipitable c.p.m. The tracks showing immunoprecipitates (8-14) were loaded with the total recovery from immunoprecipitation reactions which contained 3 x 10^6 c.p.m. (tracks 8 & 9) and 5 x 10^6 c.p.m. (tracks 10-14). Molecular weights are shown x 10^{-3}.

digests indicated that Ad2 and HSV sequences remained juxtaposed in many inserts. Most cell lines appeared to contain several independent inserts of transforming DNA sequences, and many inserts were present in multiple copies. Intact copies of both Ad2 Bam C and HSV Bam TK fragments were detected in the majority of co-transformants generated using trimers or higher order concatamers. However the amounts of such Bam Hl fragments yielded by the three Ad H lines were generally rather low suggesting that integration of long concatamers was uncommon and that more often only a small section of the concatamer became integrated. The high copy number of many inserts indicates that an amplification step frequently occurred at some stage after association of transforming DNA with the sequences which flank this in the co-transformants. The overall stable pattern of multiple inserts represented in high copy num-

bers is consistent with current views of early events in transfected cells which involve ligation of carrier DNA and transforming DNA (36) followed by amplification and integration into the cell chromosome (37).

Having characterised the virus DNA inserts in these cells we turned our attention to the cytoplasmic polyadenylated RNA populations. In order to survive in the selection medium the cells must express HSV TK and indeed all transformants contained a major RNA species which hybridised to the HSV Bam TK probe and which was present also in HSV-infected cells but was absent from uninfected untransformed cells. The fact that this RNA species was identical in size in all transformants and in addition co-migrated with the major infected cell species detected by the probe is suggestive that transcription of the TK gene in transformants is initiated at the normal HSV transcription initiation site. This is curious as transcription of this gene in infected cells is not initiated unless functional virus immediate early proteins are available (38,39). (An alternative explanation, that primary transcripts, containing different lengths of upstream sequences in different lines, are all processed to yield similarly sized mRNAs, seems unlikely but cannot be excluded by our data).

In several cell lines (the three Ad H lines) there was clear evidence that incorporated Ad2 sequences were also transcribed. The polyadenylated RNA species detected were relatively large (in excess of 2.4 Kb) but differed in size in each cell line. However they were within the approximate size range of authentic late Ad2 mRNAs detectable by the Ad2 Bam C probe. The Ad2 DNA sequences in the co-transformants included neither the late transcription promoter nor the three leader sequences so transcripts found in these cells could not correspond exactly with infected cell species. Rather it seems likely that these transcripts would be initiated in upstream sequences flanking the Ad2 DNA insert. None of the RNA species observed contained HSV sequences detectable on the autoradiogram shown in Figure 5B. However this autoradiogram was a shorter exposure than that required to visualise Ad2-specific transcripts, as the major HSV-specific RNAs in the transformants were readily detected. These major HSV TK-specific RNA species almost certainly contain no Ad2 sequences but it remains possible that the Ad2-specific transcripts contain HSV sequences not detected in this experiment. Non-virus sequences of cellular or carrier origin may also comprise part of these A2-specific RNA species.

The transcripts containing Ad2 sequences which we detected were all polyadenylated and one possibility which we considered was that these RNA species

were terminated at the polyadenylation site for L2 late Ad2 mRNAs. However using an Ad2 Hind III A probe it was shown that the transcripts from two of the three H lines contained Ad2 sequences downstream from this site. This probe also hybridised to the co-migrating RNA species in Ad H2 and Ad D1 which had been obscured by a non-specific band in blots probed with Ad2 Bam C.

Whether any of the transcripts detected contain Ad2 sequences which actually span the polyadenylation site has not yet been determined but it is quite possible that this site may not be recognised in the absence of early Ad2 proteins. This may also be true for any splice acceptor sites contained in these transcripts as it is known that the processing of Ad2 transcripts initiated at the major late promoter varies according to the stage of the infectious cycle (32,40). These questions are currently under investigation.

Moreover our data do not allow us to define the direction of transcription of the Ad2 inserts and it is possible that the template for transcription was in some cases the complement of the normal late template. We have no information on sequences in this strand which could regulate the transcription process.

Clearly however integrated Ad2 sequences from the late region of the genome can be transcribed in the absence of the late Ad2 promoter and leader sequences and the transcripts are sufficiently stable to survive processing and transport to the cytoplasm where they are detectable as polyadenylated species of defined size. However we have so far been unable to obtain any evidence for expression of these Ad2 sequences at the polypeptide level.

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