Characterization of Transforming Viruses Rescued from a Hamster Tumour Cell Line Harbouring the v-src Gene Flanked by Long Terminal Repeats

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SUMMARY

The organization of proviruses derived from infecting transforming viruses rescued from hamster tumour cells was studied. Southern blot analysis indicated that the provirus from the F6 cell line was organized as long terminal repeat (LTR)-src-LTR, and S1 mapping experiments suggested that it was probably derived by reverse transcription of src mRNA followed by integration. In the E6 cell line, the provirus unit was arranged as LTR–Δ gag–src–LTR, indicating a recombination event between the rescued transforming virus and the helper virus. These results suggest that transforming defective viruses containing only the src gene can be rescued from non-permissive mammalian cells.

INTRODUCTION

Mammalian cells can be transformed by avian sarcoma viruses and these cells often are non-permissive in that no virus is produced. However, complete virus can be rescued by fusion with permissive chicken cells (Svoboda & Hlozanek, 1970; Boettiger, 1974) implying that intact proviruses are integrated in these cells and therefore these are referred to as virogenic cells. In some cases, cell fusion is not sufficient to rescue virus even if non-defective virus strains are used to transform the mammalian cells. In these special situations, transforming virus could be obtained by fusion with chicken cells which had been pre-infected with a replication-competent helper virus such as Rous-associated avian leukosis virus (RAV-1) (Popovic et al., 1977; Steimer & Boettiger, 1977). The rescued virus, which contains both the helper virus and the transforming virus, can be used to induce tumours in mammalian hosts and characterization of these tumour cell lines has indicated the presence of defective transforming proviruses. We have previously reported the establishment of hamster tumour cell lines and basic characterization of some of the defective proviruses including the proviral structure consisting of long terminal repeat (LTR)–src–LTR sequences (Svoboda et al., 1983; Geryk et al., 1984). In this report we present evidence that such a proviral structure may have arisen from reverse transcription from the src mRNA and then re-integration. We also describe conditions for successful rescue of transforming viruses from this cell line and the structure of the rescued virus genomes.

METHODS

Cells and viruses. Avian leukosis virus-free fibroblasts were derived from 10-day-old Brown Leghorn embryos (BLEF) whose phenotype is C/E, and from 8-day-old Japanese quail embryos (QEF) with phenotype Q/BC. The origins of the chicken and quail embryos and cultivation of embryonic cells were as described by Hajkova et al. (1982). RAV-1 was kindly supplied by Drs P. M. Biggs and L. N. Payne (Houghton Poultry Research Station, Huntingdon, U.K.) in 1968, and we used virus progeny from the first passage of the original frozen virus stocks. For some experiments, RAV-1 was recloned by terminal dilution. The hamster tumour cell line H-19 which harbours the cryptic proviral LTR–src–LTR sequence was described previously (Svoboda et al., 1983; Geryk et al., 1984).


**Virus tests and virus rescue procedures.** Transforming viruses were assayed by focus assay in 6 cm polystyrene tissue culture dishes (Kooh-i-noor, Hardtmuth, Dalecin, Czechoslovakia) using the schedule described earlier (Svoboda et al., 1968) and expressed as focus-forming units (f.f.u.). Virus rescue procedures were performed as described previously (Svoboda, 1981).

**Isolation of foci and transformation of QEF.** Individual foci visualized under a microscope were picked with a Pasteur pipette and grown in Dulbecco's modification of Eagle's MEM (Gibco) supplemented as described by Murphy (1977). Cells were transferred to microplate dishes (Micro Test II, Falcon). When approaching confluence, cells were transferred to 6 cm Petri dishes. After 4 to 8 weeks of cultivation, the cultures of transformed cells were frozen in liquid nitrogen.

For transformation of QEF, frozen cells were thawed, irradiated (7000 R), mixed with QEF (1:10 to 1:100) and overlaid with agar medium. Ten days later, when foci of transformed QEF appeared, the agar overlay was removed and the cultures were passaged and grown in roller bottles until fully transformed.

For isolation of non-producing cells, QEF were infected with terminal dilutions of rescued virus. Individual foci were picked, subcultured in F10 medium supplemented with 5% calf serum, 1% chicken serum, 10% tryptose phosphate broth (Difco) and 0.5% DMSO and checked for the absence of a helper virus using the 16Q complementation assay (Geryk et al., 1984).

**Detection of proviral sequences in cellular DNA.** High molecular weight DNA was isolated from various cell lines (Svoboda et al., 1983) and digested with appropriate restriction enzymes under conditions specified by the suppliers. The DNA was fractionated on agarose gels, blotted onto cellulose nitrate filters and hybridized with 32P-labelled DNA as described in the legends. Details of nick translation and hybridization conditions have been published (Guntaka et al., 1980; Svoboda et al., 1983). Radiolabelling of various viral DNA fragments cloned in pBR322 was accomplished by nick translation using [32p]dCTP as described by Rigby et al. (1977).

**S1 mapping.** S1 mapping was done essentially as described before (Mitsialis et al., 1983) using the src-14 fragment that was labelled at the NeoI site at nucleotide 7127 (see Fig. 1). The src-14 fragment containing plasmid supercoiled DNA was digested with NeoI, dephosphorylated by bacterial alkaline phosphatase and labelled at its 5' ends with 32P by using polynucleotide kinase and [γ-32P]ATP (ICN Chemicals). About 50000 to 100000 c.p.m. (sp. act. 106 to 1.5 × 106 c.p.m./pmol) of the probe was incubated with 10 to 20 μg total RNA and hybridized at 52 to 53 °C, after which they were treated with S1 and analysed on a sequencing gel (Mitsialis et al., 1983).

**RESULTS**

**Rescue of transforming viruses from H-19 cells**

When irradiated H-19 cells were fused with RAV-1-pre-infected BLEF and seeded under an agar overlay, few foci of transformed cells appeared (Table 1). There was no substantial difference in the efficiency of virus rescue between H-19 cells cultured for short (1 month) or long (1 year) periods after cloning. Also, there was no difference in the number of foci whether 10⁶ or 10⁵ H-19 cells were used, agreeing with our previous observations that the virus rescue frequency does not increase proportionally if the ratio of mammalian to avian cells is higher than 1:60 (Svoboda & Dourmashkin, 1969).

In all individual foci picked, both rounded transformed cells and fibroblasts were seen; the nine best growing foci were selected for further analysis. Karyological analysis of progeny cells from each focus indicated typical chicken metaphase chromosomes (Table 2). The cells produced very little (7 to 490 f.f.u./ml) virus as measured by the focus assay. After a longer cultivation, higher amounts of virus were detected, reaching 4.2 × 10⁵ f.f.u./ml in the case of focus F6(E).

**Characterization of v-src of rescued virus**

Virus progeny from individual foci obtained were used to transform QEF. The DNAs from independently transformed cultures were isolated and restricted with EcoRI. The digests were fractionated on agarose gels, blotted and hybridized with nick-translated plasmid containing the 3.1 kb EcoRI fragment which has the entire src gene and a majority of the U3 region (6141 to 9293; Fig. 1). DNA from several foci showed a distinct src-containing fragment of 2.65 kb (Fig. 2: F6, G8, E7) that co-migrated with the band from the parental H-19 cell line (Fig. 2, H-19). In E6, however, the band was about 3.7 kb in size. In all these lines, including RAV-1-infected as well as normal QEF cells, a slow migrating band of about 25 kb and another fragment of 1.1 kb were also evident, which were derived from endogenous c-src of QEF and 3' end of RAV-1,
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Fig. 1. Restriction enzyme map of pATV-8 (Katz et al., 1982). The numbers next to the restriction enzyme sites refer to the nucleotide number at which the enzyme cleaves, and were taken from Schwartz et al. (1983). Solid bars indicate the fragments that were used as probes in this study. B, BamHI; R, EcoRI; P, PvuI; S, SphI; Nr, NarI; N, NdeI; NC, NcoI; H, HindIII; K, KpnI; X, XhoI; Sc, SacI; Bg, BglII.

Table 1. Rescue of transforming virus by fusion of H-19 cells with RAV-1-pre-infected BLEF

| No. of H-19 cells | H-19(S)† × BLEF RAV-1 | H-19(E)‡ × BLEF RAV-1 || H-19K1(M)§ × BLEF RAV-1 || |
|------------------|----------------------|-------------------|-------------------|-------------------|
| tested           |                     |                   |                   |                   |
| 10⁶              | 2-5                  | 2                 | 0-5               |
| 10⁵              | 3-5                  | 2                 | 0-5               |

* Two × 10⁶ BLEF seeded with indicated numbers of H-19 cells (irradiated with 7000 R using a 60Co source) were fused the next day with polyethylene glycol and overlaid with medium containing 0.8% agar (Svoboda, 1981). Number of foci is expressed as average of two dishes. Fusion of the respective number of H-19 cells with uninfected BLEF or fusion of RAV-1-infected BLEF did not produce foci.
† Cells stabilized for growth in tissue culture for more than 1 year.
‡ Cells grown from the tumour explant for 1 month.
§ Clonal cell line.
|| Recloned RAV-1 was used.

Table 2. Production of transforming virus in cell progeny of foci obtained after fusion of H-19 cells with RAV-1-infected BLEF

<table>
<thead>
<tr>
<th>Designation of subcultured focus*</th>
<th>Karyotype†</th>
<th>F.f.u./ml at</th>
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<tr>
<td></td>
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<td>1 week</td>
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<tr>
<td>A2(S)</td>
<td>Chicken</td>
<td>15</td>
</tr>
<tr>
<td>A6(S)</td>
<td>Chicken</td>
<td>45</td>
</tr>
<tr>
<td>A8(S)</td>
<td>Chicken</td>
<td>7</td>
</tr>
<tr>
<td>E6(S)</td>
<td>Chicken</td>
<td>7-5</td>
</tr>
<tr>
<td>E7(S)</td>
<td>Chicken</td>
<td>0</td>
</tr>
<tr>
<td>F1(S)</td>
<td>Chicken</td>
<td>NT</td>
</tr>
<tr>
<td>F6(E)</td>
<td>Chicken</td>
<td>490</td>
</tr>
<tr>
<td>G8(M)</td>
<td>Chicken</td>
<td>NT</td>
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* Individual foci from Table 1 were isolated. See footnotes to Table 1 for explanation of symbols (S), (E) and (M).
† Fifty metaphases were evaluated.
‡ NT, Not tested.
Fig. 2. Southern blot analysis of proviruses from different cells. High molecular weight DNAs were prepared from each cell line, digested with EcoRI, fractionated on agarose gel, transferred to the cellulose nitrate filter and hybridized with the 3.1 kb EcoRI fragment (6141 to 9293). HindIII fragments (23.1, 9.4, 6.7, 4.3, 2.3, 2.0, 0.56 kb) were used as markers (right side markings). H, Hamster cell DNA; XC, rat cell line derived from a tumour induced by avian sarcoma virus. The heavy arrows on either side indicate the position of loading wells.

respectively. Since the probe contained the U3 region of the LTR, we expected and observed hybridization to the 1.1 kb fragment of RAV-1. These bands were absent in H-19 and H-20 cell lines because these hamster cells lack RAV-1 and endogenous c-src is not highly related to the avian src probe. That the rescued virus from hamster cells contained both transforming as well as the helper RAV-1 sequences was shown by hybridizing the same blot with a pol-specific probe (data not shown).

**Analysis of the provirus unit in E6**

Because the above results suggested that the provirus in E6 might have arisen as a result of recombination between the helper RAV-1 and src gene, further studies were undertaken to characterize it more precisely. Restriction enzyme digestion followed by Southern blotting and hybridization to the src-11 probe detected an EcoRI fragment of 3.7 kb (Fig. 3a, lane 2). Hybridization of the same blot with the gag-specific probe indicated that the 3.7 kb fragment contained both gag and src sequences (data not shown). Double digestions with EcoRI and BamHI (lane 3) and NdeI (lane 5) indicated that BamHI (at nucleotide 532) reduced its size to
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Fig. 3. Analysis of proviruses of E6. (a) DNA digested with EcoRI (lane 2), EcoRI and BamHI (lane 3), BglII (lane 4) or NdeI (lane 5), blotted and hybridized with src-11. EcoRI or SphI digests of pATV-8 were used as markers in lanes 1 and 6, respectively. (b) EcoRI-BglII double digestion of RAV-1 DNA (lanes 2, 4, 6, 8) or E6 DNA (lanes 3, 5, 7, 9). As a marker, pATV-8 DNA was digested with EcoRI (lane 1). The filters were hybridized with either src-11 (lanes 1 to 3) or src-11 and src-14 (lanes 4 and 5) or gag (lanes 6 and 7) probes. Lanes 8 and 9 are the same as 6 and 7, except that they were washed and then re-hybridized with the src-14 probe. Arrows in (a) show the fragments derived from the E6 provirus.

3.1 kb whereas NdeI (at 1290) did not cleave, suggesting that the left boundary of the deletion or recombination event took place in a sequence between 532 and 1290. BglII which cleaves at 7736 gave a fragment of 1.5 kb which exclusively hybridized to the src-11 probe (lane 4) but not to src-14 (data not shown).

The E6 v-src-containing fragment was further characterized using EcoRI and BglII double digestions (Fig. 3b). As shown above (Fig. 3a, lane 4), the src-11 probe, which detects the v-src sequence downstream from the BglII site, detected only the 1.5 kb fragment in E6 (Fig. 3b, lane 3). Re-hybridization of the same filter with src-14 which detects the 5' end of the src gene, detected a new fragment of 2.3 kb (lane 5), which also includes gag sequences (Fig. 3b, lane 7) in addition to the EcoRI-BglII fragment of 1.7 kb from RAV-1 (lanes 6 and 7). To prove conclusively that the 2.3 kb fragment contained both gag and src sequences, the filter was hybridized sequentially to the gag probe and the src-14 probe (lanes 7 and 9). Other experiments showed that the 2.3 kb fragment did not hybridize with probes detecting pol or env viral genes (data not shown). It should be pointed out that in E6, in addition to this provirus unit, some other anomalous provirus units appeared to be present as evidenced by hybridization at low intensity to other fragments.

Additional experiments with an LTR-specific probe and other restriction enzymes such as PvuI, which cleaves only within the LTR, indicated that in E6 the provirus appeared to be organized as LTR-gag-src-LTR. Cloning and sequencing should reveal the arrangement of this unit.
Fig. 4. Analysis of F6 clones. DNAs were restricted and analysed as in Fig. 2. Lanes, 1, 10, 11 and 20 contain the marker, pATV-8, which was digested with EcoRI. H-19 DNA digested with EcoRI (lanes 2 and 12) or EcoRI plus SacI (lanes 3 and 13) or BamHI (lanes 4 and 14); F6 DNA digested with EcoRI (lanes 5 and 15) or EcoRI plus SacI (lanes 6 and 16) or BamHI (lanes 7 and 17); F6-NP DNA digested with EcoRI (lanes 8 and 18) or PvuI (lanes 9 and 19). Note that the PvuI fragment (lanes 9 and 19) appears to be slightly bigger than the EcoRI fragment (lanes 8 and 18). This is because PvuI does not digest cellular DNA very well as a result of which the fragment is slightly retarded due to viscosity. Digestion with another enzyme prior to digestion with PvuI reduced viscosity and the EcoRI and PvuI bands are identical (data not shown). Src-11 or gag probes, left and right panels respectively, were used for hybridization.

Analysis of the provirus in H-19, F6 and F6-NP

To demonstrate that F6 and F6 non-producing (F6-NP) cell lines contain a provirus identical to H-19, cellular DNA was isolated from H-19, F6 and F6-NP (subclone K1, F6-NPK1) cells treated with various restriction enzymes (see Fig. 1 for map positions), separated on an agarose gel, and blotted and hybridized with the src-11 and gag probes (Fig. 1). The results (Fig. 4) show that, as observed before, EcoRI yielded a fragment of 2.65 kb from H-19 (lane 2), F6 (lane 5) and F6-NPK1 (lane 8) which was reduced to about 2.3 kb upon digestion with SacI (lanes 3 and 6), whereas BamHI digestion did not alter the size of the EcoRI fragment, indicating the absence of any BamHI sites (lanes 3 and 6). Hybridization of the same blot with the gag probe detected no sequences related to gag (positions 532 to 1916) (lanes 12 to 14) in H-19 cells, whereas F6 cells which contained the helper RAV-1 as well, yielded the expected 2.4 kb EcoRI fragment which was reduced to 2.15 kb with SacI and 1.4 kb with BamHI (lanes 15 to 17). The gag sequences were not detectable in F6-NPK1 (lanes 18 and 19). Other experiments as well as unpublished data indicated that sequences related to pol and env were not present in these cell lines. The absence of all BamHI sites confirms earlier results. Since the SacI site at 255 (Fig. 1) was present but not the BamHI site at 532, this suggested, but did not prove, that the provirus probably originated from the spliced src mRNA by reverse transcription of src mRNA followed by integration. The size of the provirus in F6 and F6-NPK1 cells, as analysed by EcoRI and PvuI (the latter has two sites, one in each LTR) was exactly the same size as src mRNA from H-19 cells.

Further support for the integration of a reverse transcript of src mRNA was provided by additional digests. XbaI and XhoI, which cleave at nucleotides 6861 and 6980, respectively, did not generate the expected XbaI-BglII and XhoI-BglII fragments (data not shown) indicating that SacI (Fig. 4), XbaI and XhoI sites were not present. PstI, which cleaves at multiple sites,
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Fig. 5. Analysis of F6-NPK1, H-19, and QEF DNA. DNAs were digested with (a) PstI, (b) BglII and PstI or (c) BglII and NcoI and hybridized with the src-14 probe. Arrows indicate the positions of λ HindIII markers which are 2.3, 2.0 and 0.56 kb from top to bottom. A to F are markers of pATV-8 corresponding to 1.6, 1.25, 0.96, 0.68, 0.42, 0.39 kb, respectively. Some bands contain more than one fragment, e.g. C should represent 0.91 and 0.96 kb fragments and band D represents 0.68 to 0.62 kb; E also contains a 0.4 kb fragment and F contains 0.29 to 0.32 kb fragments.

should have generated a fragment of 397 bases if the sites at nucleotides 7048 and 7445 had been present. If the PstI site at the splice acceptor site (7048) was missing and the site at 263 in the leader sequence was intact, then we expected a fragment of 0.54 kb. The results presented in Fig. 5(a; F6-NPK1) clearly show a band around 0.56 kb but not 0.4 kb. Since the probe used was src-14, which recognized another PstI fragment of 0.6 kb (sites at 7450 and 8050), the band we detected as 0.56 kb might have contained fragments of 0.54 and 0.6 kb. This was indeed the case as proved by double digesting the DNA with BglII (7736) which split the fragment derived from positions 7450 to 8050, and left the other fragment undigested. Double digestion with PstI and BglII gave an additional broad band migrating at about 0.3 kb. We expected two bands of 0.31 and 0.29 kb. As another control, we used NcoI which has a site at the first ATG codon of the src gene. BglII-NcoI double digestions yielded the expected 0.61 kb fragment. These results strongly support the hypothesis that the provirus is derived by integration of a src mRNA reverse transcript.

S1 mapping provides further evidence for this notion. The src-14 clone was cleaved with NcoI (single site at 7124), labelled, and hybridized to total RNA isolated from H-19, F6-NP, E6, XC and Pr-C cells under conditions that favoured RNA–DNA hybridization. Following annealing, the hybrids were treated with S1 and the resistant fragment was analysed on a sequencing gel. The results (Fig. 6) clearly show protection of an 81 to 82 nucleotide fragment which was exactly the size expected for a spliced messenger. Identical results were obtained with RNA from XC
Fig. 6. S1 mapping of src-specific RNA. RNA was isolated from various cell lines and hybridized with end-labelled DNA as described in Methods. S1-resistant hybrids were analysed on a sequencing gel. *HaeIII* digested pBR322 DNA fragments were used as markers.

cells. However, in XC and Pr-C infected cells, an additional band at 262 nucleotides was observed. This was due to the protection by 35S RNA present in these cells but not in H-19, E6 and F6-NP. These results suggested that the provirus is generated from a processed src mRNA because F6 and F6-NP had been established from rescued virus and therefore only RNA correctly spliced had been encapsidated.

**DISCUSSION**

Our results document for the first time the successful rescue of v-src from mammalian cells harbouring a defective provirus, which is organized as LTR-src-LTR. Previous attempts to obtain transforming virus after fusion of similar mouse (Svoboda et al., 1971) or rat (Hughes et al., 1978) cells with chick embryo fibroblasts infected by RAV-1 or transformation-defective avian sarcoma virus (ASV) mutants failed. This might be due to the experimental procedures
used in which only free transforming virus in the supernatant of heterokaryons was measured. Our present data show that foci isolated after virus rescue initially produce very little transforming virus. A provirus rescued from QEF transformed with highly deleted Rous sarcoma virus (RSV) provirus (rdBK303) with a genome organization similar to the one described here was studied by Martin et al. (1979, 1980). However, the rdBK303 provirus does contain some env sequences which might contribute to homologous recombination with the helper virus during repeated cycles of cell transformation. In this case, the rescued transforming viruses were non-defective and therefore might have arisen as a result of recombination with helper virus. In this regard, they are similar to our E6.

The low efficiency of virus rescue and initially poor replication of rescued viruses recorded in our experiments, as well as the low level of virus rescue from QEF harbouring a similar defective provirus (Mason et al., 1979), may be caused by several factors. The cryptic provirus lacks the normal packaging signal (Pugatsch & Stacey, 1983) which decreases the chance of its transcript being packaged in a helper virus virion.

The proviral structure of transforming viruses rescued from H-19 cells falls in two categories. The majority of such viruses retained the same EcoRI src-containing fragment as that present in H-19 cells. Using one of these rescued viruses, F6, non-producer clones of transformed quail cells were obtained, which again harbour the same EcoRI src-containing fragment of 2-65 kb as the original H-19 cells and the EcoRI junction fragments are of different sizes in each non-producing clone. These findings support the possibility that F6 virus arose in a heterokaryon by the incorporation of src mRNA in the helper virus virion. After such a heterozygous particle infects a sensitive cell, the src mRNA is reverse-transcribed by helper virus reverse transcriptase and integrated at random in the host cell genome. This mechanism of viral oncogene transmission via incorporation of its transcript in a retrovirus virion may be similar to the case of the myc oncogene of MH2 virus (D. Stehelin, personal communication). Other groups have described the packaging of cellular mRNA in RSV (Adkins & Hunter, 1981) and a subgenomic env mRNA in an avian retrovirus (Stacey, 1980). In addition, env mRNA, incorporated into env gene-deficient BH-RSV(−) virions recombines, probably during reverse transcription, with BH-RSV(−), giving rise to a non-defective env gene-containing helper virus (Wang & Stacey, 1982).

Rescued E6 virus clearly differs from other rescued viruses because it acquired 1.1 kb of DNA inserted between the LTR and the 5′ end of v-src. Virus gene-specific probes show that this DNA includes about 0.6 kb from the 5′ end of the gag gene. We do not know as yet whether the remaining 0.5 kb is also of gag origin, or whether it is an insert of cellular DNA. In this context, it should be mentioned that regions of homology between src and gag gene were utilized in the generation of recovered avian sarcoma viruses (Wang et al., 1984).

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