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Integration of avian sarcoma virus DNA sequences in transformed mammalian cells

(provirus/RNA tumor viruses/agarose gels/restriction enzymes)

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ABSTRACT DNA from six avian sarcoma virus (ASV)-transformed mammalian cell lines was digested with the restriction endonucleases *Eco*RI, *Xho* I, or *Sal* I, fractionated by agarose gel electrophoresis, transferred to nitrocellulose filter strips, and hybridized with specific ASV [^{32}P]cDNA probes. DNA from all of the ASV-transformed cell lines yielded three common virus-specific DNA fragments (2.4, 1.8, and 1.3 × 10^6 daltons) upon cleavage with *Eco*RI. *Xho* I appeared to cleave at least once within the integrated provirus and yielded a common fragment of 3.3 × 10^6 daltons as well as a second virus-specific DNA fragment whose size varied from 4.0 to 5.0 × 10^6 daltons in the different transformed cell lines. *Sal* I did not cleave within the provirus and yielded a single major virus-specific fragment of about 11 × 10^6 daltons in all transformed lines examined. Using specific cDNA probes, we show that the 1.8 × 10^6-dalton *Eco*RI fragment contains sequences homologous to the 3′ end of the viral RNA as well as to the *src* region of the viral genome. These studies clearly demonstrate that the same region on the ASV genome is utilized for provirus integration in different ASV-transformed cell lines.

A central observation in the study of DNA and RNA tumor viruses is the persistence of viral genetic information in the host genome of the transformed cell (1, 2). Transformation of mammalian cells by the DNA tumor virus simian virus 40 (SV40) involves the covalent insertion of the viral DNA into the host cell genome (3). Recently, it has been shown that the arrangement of the integrated SV40 DNA sequences is different in independently isolated clones of SV40-transformed cells (4, 5). These results imply that the site of SV40 DNA integration in the cellular genome is not unique and that the SV40 sequences involved in integration are not confined to a specific region of the viral genome (4, 5). Transformation of mammalian cells by RNA tumor viruses requires the formation of a DNA copy (provirus) of the viral RNA and the integration of the provirus into the host genome (2). Molecular hybridization studies have shown that avian sarcoma virus (ASV)-transformed mammalian cells contain one to two copies of covalently integrated provirus per cell and that normal mammalian cells contain little if any DNA sequences homologous to the ASV genome (6–8).

To determine the arrangement of integrated proviral sequences in ASV-transformed mammalian cells, DNA from six ASV-transformed mammalian cell lines has been digested with restriction endonucleases *Eco*RI, *Xho* I or *Sal* I, fractionated by agarose gel electrophoresis, transferred to nitrocellulose filter strips, and hybridized to labeled cDNA probes complementary to either the entire ASV genome or to nucleotide sequences at the 3′ or 5′ end of the viral RNA.

Comparison of the viral-specific DNA fragments generated by these restriction enzymes and the various [^{32}P]cDNA probes has led us to conclude that the same region on the ASV genome is involved in provirus integration in the different ASV-transformed cell lines.

METHODS

Viruses, Cells, and Preparation of Cellular DNA. Procedures for the purification of virus and growth of cells in culture have been described (9, 10). 3T3 (A31) cells were obtained from the American Type Culture Collection. Bratislava strain (B_{77})/3T3, Schmidt–Ruppin (SR)/3T3, and B_{77}/normal rat kidney (NRK) cell lines were kindly provided by P. K. Vogt. SR/BALB/c and SR/C_{57} mouse cell lines were provided by D. P. Bolognesi. B_{77} strain ASV was obtained from R. Smith. AMV and RNA-dependent DNA polymerase were provided through the office of Program Logistics, National Cancer Institute. High-molecular-weight DNA was purified from normal and transformed cells essentially as described by Varmus *et al.* (6).

Restriction Enzymes, Agarose Gels, and Blotting Technique. *Eco*RI, *Xho* I, and *Sal* I restriction endonucleases were purchased from New England Biolabs. Enzyme digestions (11–13) were done at DNA concentrations of 40–80 μg/ml at 37° for 2 hr. In all experiments, complete digestion of cellular DNA preparations was monitored by adding 2 μg of λ DNA to one of the samples of cellular DNA. Gel electrophoresis was carried out in vertical slab gels (21 × 18 × 0.6 cm) in 1% agarose (Seakem), for 23 hr at 55 V in a buffer described previously (4). The DNA in the gels was denatured, neutralized, and transferred to nitrocellulose strips essentially as described by Southern (14). Efficiency of transfer was monitored by measuring the amount of λ [^{32}P]DNA fragments retained on nitrocellulose strips or by restaining the gels with ethidium bromide after transfer.

Preparation of [^{32}P]cDNA Probes. ASV RNA was prepared from purified preparations of virus as described (9). [^{32}P]cDNA probes complementary to the entire viral genome or to the 5′ and 3′ ends of ASV RNA were prepared as described in the figure legends.

Hybridization to Nitrocellulose Strips. After blotting, nitrocellulose strips (Schleicher and Schüll) were washed and incubated in preincubation buffer (15) for 16 hr at 68°. [^{32}P]-cDNA probe, sodium dodecyl sulfate (final concentration, 0.5%), and yeast RNA (20 μg/ml) were then added to the hybridization mixture. Hybridization was carried out for 72–96 hr at 68° and the filter strips were then washed as described by Botchan *et al.* (5). Filter strips were dried and subjected to autoradiography (Kodak film XRP-5) for 3 days to 3 weeks.

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Abbreviations: SV40, simian virus 40; ASV, avian sarcoma virus; B_{77}, Bratislava strain ASV; SR, Schmidt–Ruppin strain ASV; NRK, normal rat kidney; XC, Prague C-transformed rat cells; AMV, avian myeloblastosis virus.

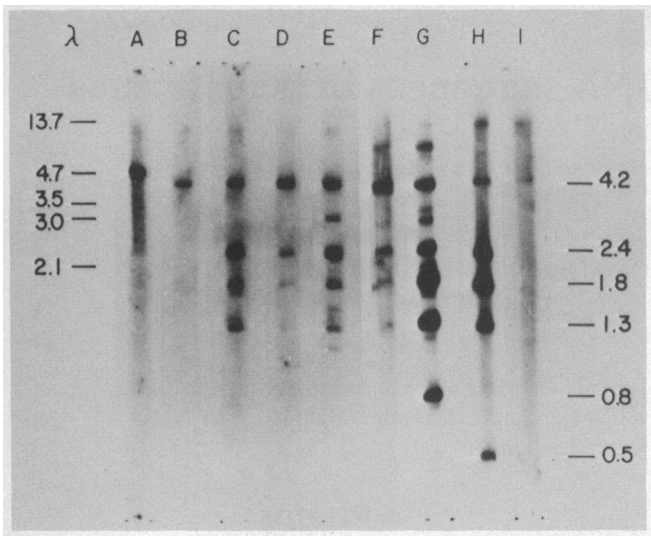

FIG. 1. Detection of ASV-specific sequences in *Eco*RI-digested cellular DNA. DNA (20 μg) was digested to completion with *Eco*RI, electrophoresed, and transferred to nitrocellulose strips. The preparation of [^{32}P]cDNA representative of virtually the entire ASV genome was carried out in a 0.4-ml reaction containing: 40 mM Tris·HCl (pH 7.6); 30 mM NaCl; 5 mM $MgCl_2$; 0.6 mM dithiothreitol; 0.1 mM dATP, dGTP, and TTP; 0.4–0.6 μM [^{32}P]dCTP (Amersham/Searle or New England Nuclear) (specific activity, 130–250 Ci/mmol); 20 μg of actinomycin D; 15 μg of purified B_{77} ASV 70S RNA; 125 μg of calf thymus DNA fragments prepared according to Summers (19); and 180 units of RNA-dependent DNA polymerase. cDNA probes were characterized by: (*i*) hybridization of [^{32}P]cDNA with 70S B_{77} RNA, and (*ii*) hybridization of cDNA with [^{32}P]B_{77} RNA, followed by digestion with RNase T_1 and fingerprint analysis of the labeled RNA·DNA hybrids by two-dimensional gel electrophoresis (20). All of the major RNase T_1 oligonucleotides of B_{77} ASV RNA were protected by the cDNA probe and greater than 98% of labeled cDNA annealed with B_{77} 70S RNA. These experiments demonstrate that all of the ASV RNA sequences are contained in the cDNA probe although the frequency of representation may not be uniform. Nitrocellulose strips containing cellular DNA were hybridized with 12 to 15 × 10^6 cpm of [^{32}P]cDNA (specific activity, 250 to 400 × 10^6 cpm/μg) and autoradiographed. Filter strips contained DNA from (A) calf thymus, (B) 3T3, (C) SR/C_{57}, (D) B_{77}/3T3, (E) SR/BALB/C, (F) B_{77}/NRK, (G) XC, (H) B_{77}-transformed duck embryo, and (I) normal duck embryo. The positions of *Eco*RI λ DNA fragments are shown on the left. Values here and for subsequent figures represent daltons × 10^{-6}.

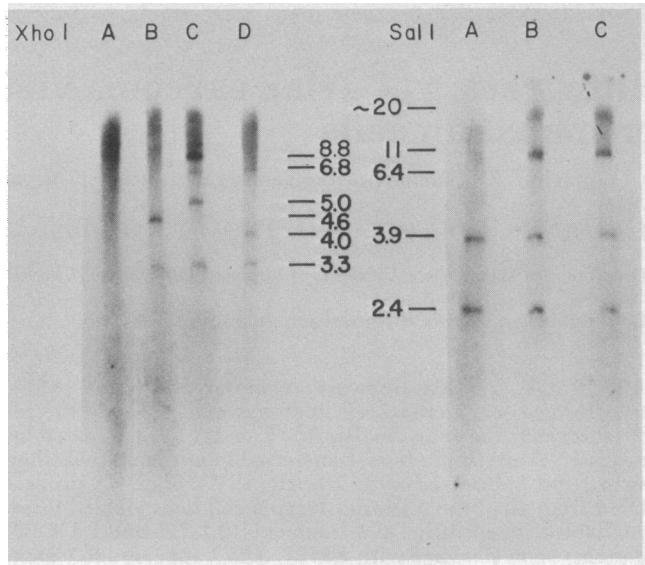

FIG. 2. Detection of ASV-specific sequences in *Xho* I- or *Sal* I-digested cellular DNA. Cellular DNA was digested, processed, and hybridized to [^{32}P]cDNA representative of the entire ASV genome as described in Fig. 1. *Xho* I-digested DNA samples: (A) 3T3, (B) B_{77}/3T3, (C) SR/BALB/c, and (D) SR/C_{57}. *Sal* I-digested DNA samples: (A) 3T3, (B) B_{77}/3T3, and (C) SR/C_{57}.

RESULTS

ASV-Specific DNA Fragments Produced by Cleavage with *Eco*RI. To examine the arrangement of integrated ASV proviral sequences in the genome of ASV-transformed cells, the following experimental approach was taken. High-molecular-weight DNA isolated from several ASV-transformed mammalian cell lines was digested to completion with a site-specific restriction endonuclease (*Eco*RI, *Xho* I, or *Sal* I). The resulting linear DNA fragments were fractionated by electrophoresis on 1% agarose gels, denatured, and transferred to nitrocellulose filter strips (14). The distribution of DNA fragments containing virus-specific sequences was determined by hybridization of the filter strips with [^{32}P]DNA complementary to the ASV RNA genome (cDNA), followed by autoradiography. Fig. 1 shows the distribution of virus-specific *Eco*RI DNA fragments in several normal and ASV-transformed cell lines. Two of the ASV-transformed cell lines (B_{77}/3T3 and B_{77}/NRK) have been shown to contain one to two viral genome copies per diploid cell (6). Digestion of calf thymus DNA or mouse 3T3 DNA yielded a single *Eco*RI band (4.8 and 4.2 × 10^6 daltons, respectively) containing DNA with sequence homology to a [^{32}P]cDNA probe representative of the entire viral RNA genome.

All ASV-transformed mammalian cell lines examined contained three common virus-specific *Eco*RI fragments (2.4, 1.8, and 1.3 × 10^6 daltons) in addition to the 4.2 × 10^6-dalton *Eco*RI fragment identified in mouse 3T3 DNA. Two of the transformed cell lines [SR/BALB/c and Prague C-transformed rat cells (XC)] contained additional virus-specific *Eco*RI DNA fragments. SR/BALB/c DNA contained a virus-specific fragment of 3.0 × 10^6 daltons. XC DNA contained a major virus-specific fragment of 0.8 × 10^6 daltons and several minor virus-specific fragments that varied from 1.2 to 6.2 × 10^6 daltons. The DNA fragment pattern observed for XC cells was consistent with the large number of proviral copies (approximately 20) found in these cells (7), and the complexity of the pattern may reflect the insertion of a number of defective viral genomes. *Eco*RI digestion of DNA isolated from duck embryo cells fully transformed with B_{77} ASV yielded four virus-specific DNA fragments, three of which were identical in mass to those observed in ASV-transformed mouse and rat cells. The fact that all of these ASV-transformed cells contained the same three virus-specific *Eco*RI DNA fragments strongly suggests that proviral sequences are inserted in the cellular genome in such a way that one always generates the same virus-specific fragments upon digestion with *Eco*RI.

ASV-Specific DNA Fragments Produced by *Xho* I and *Sal* I. To further characterize the integrated proviral sequences, DNA from normal and ASV-transformed mouse cells was digested with the restriction endonuclease *Xho* I and fractionated on agarose gels and the resulting DNA fragments were annealed with ASV [^{32}P]cDNA (Fig 2). 3T3 DNA contained several minor, high-molecular-weight *Xho* I fragments that appeared to have homology with the [^{32}P]cDNA probe. B_{77}/3T3 and SR/C_{57} DNA each contained two virus-specific *Xho* I fragments and SR/BALB/c DNA contained three virus-specific *Xho* I fragments. The 3.3 × 10^6-dalton *Xho* I fragment was common to the three ASV-transformed cell lines examined. The second virus-specific *Xho* I fragment in B_{77}/3T3 was 4.6 × 10^6 daltons and in SR/C_{57} was 4.0 × 10^6 daltons. In SR/BALB/c

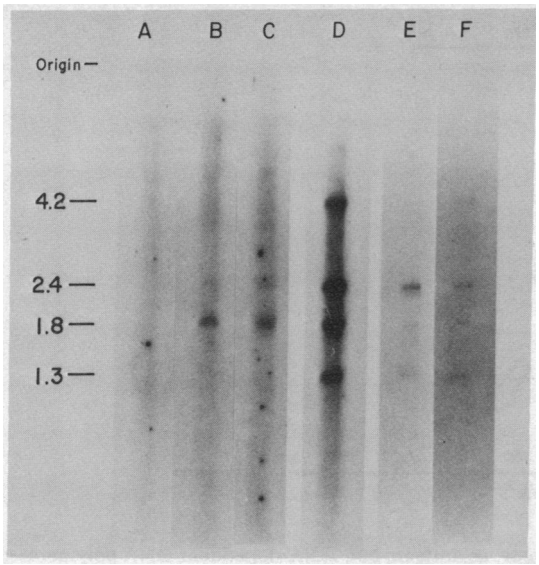

FIG. 3. Detection of ASV-specific fragments in *Eco*RI-digested cellular DNA with [^{32}P]cDNA$_3$′ or [^{32}P]$_5$′. DNA was digested and processed as described in Fig. 1. cDNA$_3$′ was synthesized by using heat-denatured ASV RNA and oligo(dT)$_{12-18}$ as template primer in the reaction mixture described in Fig. 1. cDNA$_5$′ was synthesized with the endogenous tRNA^{Trp}–viral RNA 70S template primer complex, and the cDNA products were subjected to electrophoresis on a 15% Tris·borate/polyacrylamide gel (21). Labeled cDNA about 100 nucleotides in length was recovered from the gel and used for hybridization experiments. Filters containing DNA fragments were hybridized with either [^{32}P]cDNA$_3$′ (6 to 8 × 10^6 cpm; specific activity, 200 to 350 × 10^6 cpm/μg) or [^{32}P]cDNA$_5$′ (1.0 to 1.5 × 10^6 cpm; specific activity, 350 × 10^6 cpm/μg) as described in Fig. 1. [^{32}P]cDNA$_3$′ was hybridized with DNA from (A) 3T3, (B) B$_{77}$/3T3, and (C) SR/C$_{57}$. [^{32}P]cDNA$_5$′ was hybridized with DNA from (E) SR/C$_{57}$ and (F) B$_{77}$/3T3; (D) contained SR/C$_{57}$ DNA hybridized to [^{32}P]cDNA representative of the entire B$_{77}$ RNA genome.

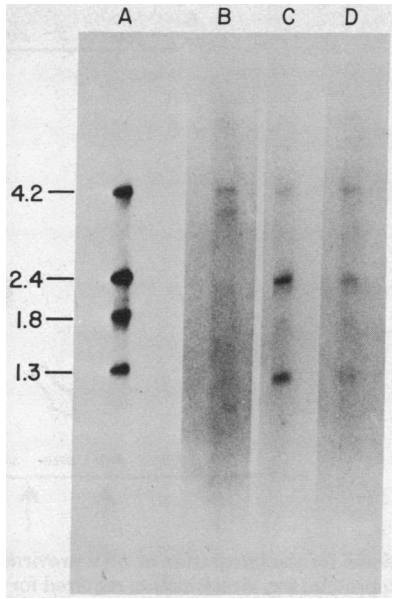

FIG. 4. Detection of AMV- and ASV-specific sequences in *Eco*RI-digested cellular DNA. DNA was digested and processed as described in Fig. 1. Labeled cDNA representative of the entire B$_{77}$ ASV or AMV RNA genome was prepared as described in Fig. 1. B$_{77}$ ASV cDNA was annealed with (A) SR/C$_{57}$ DNA. AMV cDNA was annealed with (B) 3T3 DNA, SR/C$_{57}$ DNA, and (D) B$_{77}$/3T3 DNA.

DNA, the two additional virus-specific DNA fragments were 5.0 and 6.8 × 10^6 daltons. The total mass of the two virus-specific *Xho* I fragments in B$_{77}$/3T3 (7.9 × 10^6 daltons) and SR/C$_{57}$ (7.3 × 10^6 daltons) was greater than the predicted mass of the proviral genome (6.6 × 10^6 daltons), suggesting covalent linkage of cellular DNA sequences to at least one of the *Xho* I fragments. These data also suggest that the integrated provirus contains at least one *Xho* I cleavage site. The 3.3 × 10^6-dalton virus-specific *Xho* I fragment may be generated by a second *Xho* I cleavage site located near the end of the proviral genome; it is not clear, however, whether this cleavage site is within the viral DNA sequence or within an adjacent cellular sequence.

Digestion of normal and ASV-transformed cell DNA with *Sal* I yielded the fragment pattern shown in Fig. 2. 3T3 DNA contained two *Sal* I DNA fragments of 3.9 and 2.4 × 10^6 daltons that exhibited homology with the [^{32}P]cDNA probe. DNA of two ASV-transformed mouse cell lines, B$_{77}$/3T3 and SR/BALB/c, contained two *Sal* I fragments identical in mass to those observed in 3T3 DNA. In addition, B$_{77}$/3T3 and SR/C$_{57}$ each contained a major virus-specific band of approximately 11 × 10^6 daltons and a less pronounced band of 20 to 25 × 10^6 daltons. Analysis of *Sal* I digests of transformed rat cell DNA (XC or B$_{77}$/NRK) revealed a similar virus-specific *Sal* I fragment of approximately 11 × 10^6 daltons (data not shown). Although this 11 × 10^6-dalton fragment was in the nonlinear region of the gel, it was clearly resolved from uncut DNA and from other DNA fragments differing in mass by 5 × 10^5 daltons. The position of this fragment with respect to internal markers suggests that it is essentially identical in the different ASV-transformed cell lines examined. The presence in transformed cells of only a virus-specific fragment significantly larger than the provirus suggests that integrated viral DNA sequences do not contain a cleavage site for *Sal* I.

Distribution of 3′ and 5′ Viral RNA Sequences in *Eco*RI Proviral Fragments. The location of 3′- and 5′-terminal viral sequences within the three major virus-specific *Eco*RI DNA fragments could be determined by using [^{32}P]cDNA complementary to the nucleotide sequences at the 3′ end of the viral genome (cDNA$_3$′) or cDNA complementary to the 5′-terminal 100 nucleotides of the viral genome (cDNA$_5$′). [^{32}P]cDNA$_3$′, synthesized by using heat-denatured ASV RNA and oligo(dT)$_{12-18}$ as primer, was hybridized with *Eco*RI fragments of normal and ASV-transformed cell DNA. No hybridization of [^{32}P]cDNA$_3$′ was observed with 3T3 DNA (Fig. 3). Hybridization of [^{32}P]cDNA$_3$′ with B$_{77}$/3T3 or SR/C$_{57}$ DNA showed that the 1.8 × 10^6-dalton *Eco*RI fragment contained most of the viral sequences homologous to the 3′ end of the viral RNA. Hybridization experiments with the [^{32}P]cDNA$_5$′ probe indicated that 5′-terminal ASV RNA sequences appeared to be contained within the 2.4 × 10^6-dalton virus-specific *Eco*RI fragment. However, we also observed hybridization of this cDNA$_5$′ probe to the other virus-specific *Eco*RI fragments. The hybridization pattern could be due to either contamination of the cDNA$_5$′ probe with sequences derived from other parts of the viral genome or the presence of 5′-terminal sequences repeated within the viral RNA genome. Preliminary experiments with a more highly purified cDNA$_5$′ probe indicated that this cDNA$_5$′ probe hybridized predominantly to the 1.3 × 10^6-dalton *Eco*RI fragment (data not shown). Because of the difficulty in obtaining a cDNA$_5$′ probe completely free of sequences derived from other parts of the viral genome, we cannot at this time unambiguously assign the 5′-terminal sequence to a particular *Eco*RI fragment.

Hybridization of AMV cDNA to *Eco*RI DNA Fragments. The ASV RNA genome codes for four viral specific proteins: *gag*, gs antigens; *pol*, RNA-dependent DNA polymerase; *env*,

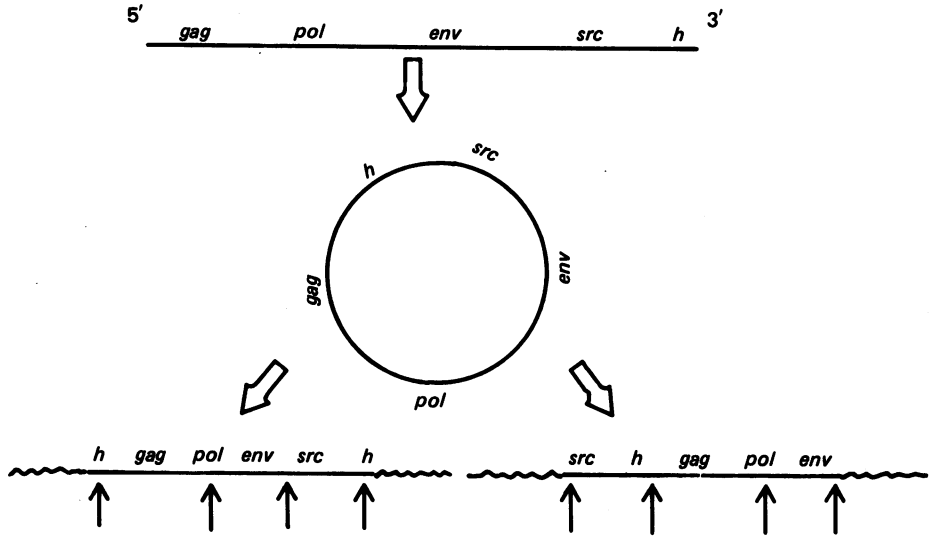

FIG. 5. Model for the integration of ASV provirus. The four virus genes are: *gag*, gs antigen; *pol*, RNA-dependent DNA polymerase; *env*, envelope glycoprotein; *src*, viral function required for cellular transformation; and *h*, heteropolymeric region common to all avian RNA tumor viruses. Arrows indicate the possible cleavage sites of *Eco*RI restriction endonuclease.

envelope glycoprotein; and *src*, the gene product required for cellular transformation. Avian leukosis viruses [including avian myeloblastosis virus (AMV)] contain the three viral genes required for virus replication but lack the nucleotide sequences specific for the *src* gene (16). To determine which of the *Eco*RI DNA fragments contains *src*-specific DNA sequences, [^{32}P]-cDNA was prepared from either B_{77} ASV RNA or AMV RNA. Because AMV RNA does not contain detectable *src*-specific sequences, virus-specific fragment(s) containing *src* sequences should show significantly less hybridization with an AMV cDNA probe compared to a B_{77} ASV cDNA probe. Fig. 4 shows that the extent of labeling of the 1.8×10^6-dalton *Eco*RI fragment by AMV [^{32}P]cDNA was significantly less than the labeling by B_{77} ASV [^{32}P]cDNA. Based on these data, we conclude that the 1.8×10^6-dalton virus-specific fragment contains most, if not all, of the *src*-specific DNA sequences.

DISCUSSION

We have used three restriction endonucleases to examine the arrangement of integrated viral DNA sequences in six different ASV-transformed mouse and rat cell lines. All of these ASV-transformed DNAs yielded three common virus-specific DNA fragments (2.4, 1.8, and 1.3×10^6 daltons) upon cleavage with *Eco*RI. Using specific cDNA probes, we found that the 1.8×10^6-dalton fragment contained sequences homologous to the 3' end of the viral RNA as well as to the *src* region of the viral genome. Hybridization experiments using $cDNA_5'$ initially showed significant annealing of labeled $cDNA_5'$ to the 2.4×10^6-dalton *Eco*RI fragment as well as to the 1.8 and 1.3×10^6-dalton *Eco*RI fragments. Further purification of the $cDNA_5'$ revealed the hybridization of $cDNA_5'$ to only the 1.3×10^6-dalton *Eco*RI fragment. *Xho* I appeared to cleave within the integrated viral DNA sequence generating one DNA fragment (3.3×10^6 daltons) that was common to the three transformed cell lines and a second virus-specific DNA fragment whose mass varied in the different cell lines examined. *Sal* I appeared not to cleave within the integrated proviral sequences and yielded a virus-specific fragment of about 11×10^6 daltons in all the transformed lines examined.

Normal mammalian and duck DNA contain sequences with homology to the ASV cDNA probe. Hybridization experiments with labeled cDNA and excess 3T3 or calf thymus DNA demonstrated only a small degree of sequence homology (1–2%) between the cDNA probe and normal cell DNA (ref. 6; P. Dierks and J. T. Parsons, unpublished data). The extent of hybridization that we observed when excess cDNA probe was annealed to restriction endonuclease fragments of normal DNA (Fig. 1) suggests the presence of cellular DNA fragment(s) containing short, repeated sequences homologous to the ASV cDNA probe. The presence of sequences complementary to $tRNA^{Trp}$ in the viral RNA genome (17) suggests the possibility that the cDNA probe may be hybridizing to repeated cellular $tRNA^{Trp}$ genes. It is not clear whether the normal cellular DNA sequences displaying viral homology are related to the site(s) of viral DNA integration.

Current evidence suggests that a double-stranded, circular DNA copy of the viral RNA is required for integration (8, 18). The presence of the same three *Eco*RI fragments in the different ASV-transformed cell lines indicates that the same site on the circular provirus is involved in recombination with the host DNA. These results are clearly different from those obtained with SV40-transformed cells, indicating that there is no unique site on the SV40 genome that is required for integration into the host DNA. Fig. 5 illustrates two possible modes of insertion of the ASV genome into the host cell DNA. If the viral insertion site is located within the heteropolymeric region (*h*), the gene order of the integrated provirus would be colinear with the viral RNA. However, if the recombination site on the circular provirus is between viral genes (for example, *src* and *env*), the gene order of the integrated provirus would not be colinear with the viral RNA. The latter arrangement of viral genes could account for the lack of virus replication in mammalian cells as well as provide for the efficient transcription of the *src* gene via a nearby cellular promoter.

Densitometry studies indicate that the three virus-specific *Eco*RI fragments are present in approximately equimolar amounts, suggesting that these fragments contain few, if any, covalently linked cellular sequences. This would place the terminal *Eco*RI cleavage sites close to the viral–cell junctures (Fig. 5). Recently, P. Shank and H. E. Varmus (personal communication) have shown that *Eco*RI cleavage of both linear and circular forms of unintegrated viral DNA (obtained from either B_{77} or Prague C ASV-infected cells) yields fragments, similar in mass to those observed in ASV-transformed mammalian cells.

Their results indicate that the terminal *Eco*RI sites are located within the viral DNA sequences.

Our experiments to date do not allow us to distinguish between integration of the ASV genome at a unique cellular site or integration at nonspecific site(s) within the host cell DNA. *Xho* I cleavage of transformed-cell DNA yielded a virus-specific DNA fragment that appeared to contain cellular DNA sequences and varied in mass in the different transformed cell lines. These data suggest that the cellular integration site as defined by the *Xho* I cleavage site is not identical in the different transformed cell lines. *Sal* I cleavage yielded a large virus-specific DNA fragment (11×10^6 daltons) that appeared to have a similar mass in the different transformed cell lines. This observation indicates that viral DNA integration may occur in similar regions of the cellular genome. Additional experiments are required to fully characterize the cellular sequences adjacent to the integrated ASV genome.

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