Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 2117–2121, April 1983
Biochemistry

Isolation of cellular DNA sequences that allow expression of adjacent genes

(eukaryotic gene expression/DNA transfection/DNA cloning/polyoma virus transformation/BAL-31 deletions)

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Communicated by Walter F. Bodmer, December 27, 1982

ABSTRACT **We have employed a strategy for the isolation and identification of cellular control (expression) sequences dependent on their ability to confer expression on a selectable gene devoid of its own expression sequences. The polyoma virus (Py) *Hae* II–*Bam*HI DNA fragment, which comprises 84% of the intact viral DNA and contains the Py transforming region but lacks Py 5′ expression sequences, was decreased markedly in its transformation of rat cells. *Hae* II-cleaved mouse cellular DNA was ligated to the Py *Hae* II–*Bam*HI fragment. A transformed colony (H1) isolated after transfection of the ligated DNA onto rat cells was found to contain multiple inserts of Py DNA, most of which were biologically inactive. A transformed colony (H2) isolated after transfection of rat cells with total H1 DNA was found to contain a single insert of Py DNA. The H2 cells are highly tumorigenic and synthesize the three Py tumor antigens. Initiation of transcription of the Py early mRNAs in H2 cells occurs at the same Py nucleotides as in complete Py DNA. The viral and adjacent cellular DNA sequences were cloned from H2 cellular DNA. The transforming efficiency of the cloned Py transforming region and adjacent H2 cellular DNA was 20–40% of that of the viral DNA containing Py expression sequences. By BAL-31 deletion mapping it was observed that the first 58 base pairs of H2 cellular DNA were sufficient for the expression of the Py-transforming region. The sequence of the first 149 base pairs of the H2 cellular DNA was determined and does not show any striking similarities to upstream 5′ sequences of a number of viral and host structural genes. Features of the H2 cellular sequence are discussed.**

Sequences 5′ to a large number of eukaryotic structural genes transcribed by RNA polymerase II have been identified as being important for gene expression. One is the "TATA sequence" found ≈30 base pairs (bp) (−30) before the initiation of transcription (1, 2). The TATA sequence appears to be involved in directing the homogeneous initiation of transcription, as its absence results in transcription starting at multiple sites (3–7). A second conserved element, the "CAAT box," has been identified in most, but not all, eukaryotic genes at about −80 (8, 9). The involvement of the CAAT box as a transcriptional signal is controversial. Deletion mapping studies have shown that the CAAT box and sequences around −80 to −100 are important for efficient expression of globin genes *in vivo* (10–12). However, McKnight and Kingsbury (13) have shown that a guanine-rich sequence between −47 and −61 and a cytosine-rich sequence between −80 and −105 are important for expression of the herpes thymidine kinase gene, but alterations to the sequence from −61 to −80, which contains a CAAT box, had no apparent effect.

In papovaviruses and retroviruses further upstream elements, called enhancers, which appear to modulate gene expression (14–22), have been identified. The enhancer elements appear to be independent of orientation and can affect expression at a distance from the gene (17, 19, 21, 22). Cellular sequences with similar properties have been isolated recently from human DNA (23). No common sequence or structural feature has yet been associated with the enhancer elements.

Thus, it would appear that different genes can use a variety of sequences to regulate their expression. In a search for cellular expression sequences we have ligated fragments of mouse DNA to a selectable gene devoid of its regulatory upstream 5′ sequences. In this report we describe the isolation and partial characterization of a rodent sequence that restores expression to the polyoma virus (Py)-transforming region lacking upstream 5′ regulatory elements.

MATERIALS AND METHODS

The Induction of Transformation and Tumor Formation. The Py *Hae* II–*Bam*HI DNA fragment, which was 84% of the intact DNA and isolated by gel purification after cleavage of 5 μg of Py DNA with *Hae* II and *Bam*HI, was ligated to 20 μg of *Hae* II-digested mouse DNA. The ligated mixture was used to transform rat-1 cells in a calcium phosphate precipitate as described (24). To test biological activity recombinant plasmid DNAs mixed with 20 μg of rat-1 DNA were used to transfect rat-1 cells and, after 2 weeks, the number of transformed foci were scored with Leishman stain (25). Tumor formation in syngeneic Fischer rats was performed as described (26).

Blotting Analysis and Nuclease S1 Mapping. Total cellular DNA was isolated as described (26). Restriction endonuclease digestions were performed on 10 μg of cellular DNA. The enzymes were purchased from New England BioLabs and were used under the conditions suggested by the manufacturer. The restricted DNA was fractionated in agarose gels and transferred to nitrocellulose paper according to the procedure of Southern (27). After transfer the filters were hybridized and processed as described (25, 26). For nuclease S1 mapping (28, 29), the procedure described by Kamen *et al.* (7) has been followed. The poly(A)$^+$ mRNAs isolated from transformed cells were hybridized to 5′ ^{32}P-labeled single-strand viral DNA fragments and the resulting hybrids were treated with nuclease S1 and fractionated on an 8% urea/polyacrylamide sequence gel (30). The *Hin*fI E-strand fragment [labeled at nucleotide (Nt) 385 and extending to Nt 5,073] of Py DNA was used as probe.

Cloning of H2 Py Insert in Prokaryotic Vectors. *Eco*RI-cleaved H2 total cellular DNA was fractionated on 10–40% su-

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Abbreviations: Nt, nucleotide; Py, polyoma virus; bp, base pair(s); kb, kilobase(s).

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crose gradients (31). Fractions were assayed for the presence of Py sequences by Southern blot analysis. Positive fractions were ligated to the purified arms of DNA from phage λgt.WESλB. Ligation and packaging of λ DNA *in vitro* was performed as described (25). Between 1×10^5 and 4×10^5 plaques per microgram of λ DNA arms were obtained, and they were screened by the methods of Benton and Davis (32). Positive plaques were picked and purified and their DNA was isolated (31). Fragments containing Py sequences were subcloned in pAT153 (33) for biological assays and DNA sequence analysis.

BAL-31 Deletion Mapping and DNA Sequence Analysis. One hundred micrograms of pB1 plasmid DNA (see Fig. 1) was cleaved with *Bam*HI, extracted with phenol, and precipitated with ethanol. The *Bam*HI-linearized pB1 DNA then was treated with BAL-31 nuclease for 1–10 min at 30°C (34). The treated DNA was ligated to *Xho* I linkers cut with *Xho* I, circularized, and cut with *Bam*HI, before transformation of competent *Escherichia coli* HB101. The extent of the BAL-31-induced deletions was determined by DNA sequence analysis. The recombinant plasmid DNAs (pB1 and the BAL-31 deletions) were subjected to sequence analysis by the chemical degradation method of Maxam and Gilbert (35). The DNAs were cut at *Nar* I sites in the pAT153 sequence and labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase (35). A secondary cut was made with *Sac* I or *Eco*RI. The SEQ computer program (36) was used for homology and symmetry analyses.

RESULTS

Isolation of Rat Cell Clones H1 and H2 Transformed as a Consequence of Cellular Regulatory (Expression) Sequences. To isolate cellular control (expression) sequences the strategy presented in Fig. 1 was utilized. Py DNA containing an intact transforming region and upstream 5′ expression sequences transforms rodent cells efficiently. If the upstream expression sequences were removed and the transforming region left intact, the transforming efficiency was decreased markedly. The *Hae* II–*Bam*HI DNA fragment (84% of the intact viral DNA) of Py DNA (Nts 95–4,632) contained an intact transforming region (Nts 174–1,560) but lacked Py expression sequences, being devoid of most of the upstream 5′ noncoding region (Nts 4,632–95) (37) (Fig. 1). The *Hae* II–*Bam*HI fragment contained about 53 bp 5′ to the major sites for the initiation of transcription of the Py early mRNAs (7). Within these 53 bp was found the Py early region TATA sequence. The Py *Hae* II–*Bam*HI fragment transformed with an efficiency 0.1–1% of that of viral DNA con-

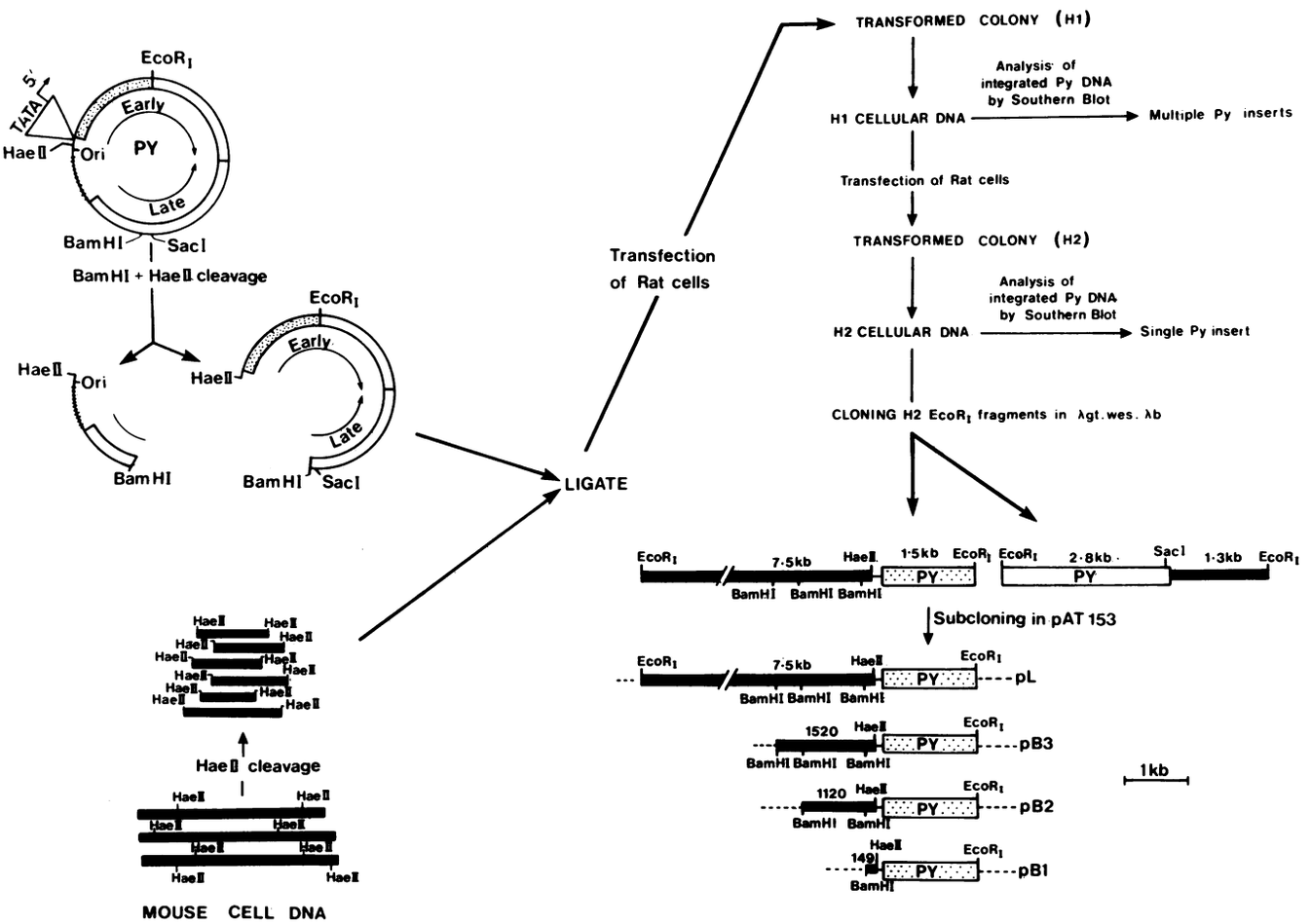

FIG. 1. Strategy for isolation of cellular expression sequences. In the upper left and lower right the Py genome is represented with the early and late coding regions boxed and the noncoding region shown as a thin line. The early coding region required for transformation is stippled and the noncoding region containing Py expression sequences is shown as a wavy line. The origin of DNA replication (ori), the TATA sequence, the initiation of transcription of the early mRNAs, and the direction of early and late transcripts are shown. The *Hae* II site at Nt 95, the *Bam*HI site at Nt 4,632, and the *Sac* I site at Nt 4,341 are indicated. Cellular DNA is represented by black bars in the lower left and lower right. In the lower right the 9- and 4.1-kilobase (kb) *Eco*RI Py-cellular DNA fragments cloned from H2 DNA into λgt.WESλB and the *Eco*RI and *Bam*HI–*Eco*RI fragments containing the Py-transforming region and various amounts of adjacent cellular DNA (7.5 kb, 1,650 bp, 1,250 bp, and 149 bp) subcloned into the plasmids pL, pB3, pB2, and pB1 are shown.

taining the Py expression sequences (see Table 1).

BALB/c mouse embryo DNA cleaved with *Hae* II was ligated to the Py *Hae* II–*Bam*HI DNA fragment (which was 84% of the intact DNA) and transfected onto rat-1 cells to induce colonies of transformed cells. Total cellular DNA from a transformed cell colony (H1) arising after transfection was analyzed for the number of inserts of viral sequences by blot hybridization. The H1 line was found to contain multiple inserts of Py DNA, as digestion with either *Bgl* II (which does not cleave Py sequences) or *Eco*RI (cleaves Py DNA once) produced multiple fragments that hybridize with a Py probe (Fig. 2). The three Py early proteins [large (M_r 100,000), middle (M_r 56,000), and small (M_r 22,000) tumor antigens] were synthesized by H1 cells (data not shown). The H1 cells were highly tumorigenic in syngeneic Fischer rats, inducing tumors within 1 month after the inoculation of 10^4 cells into adult animals.

When *Eco*RI-cleaved H1 DNA was cloned in λgt.WESλB 1 in 10,000 plaques was found to contain Py sequences. Twelve cloned H1 *Eco*RI fragments tested that contained the 5′ end of the Py early region were found to have a transforming activity that was greatly decreased. Further analysis revealed that a number of the cloned fragments lacked the *Hae* II cleavage site used for joining of viral and cellular sequences and that in many clones an intact transforming region was not present. Some of the clones contained more than one insert of viral sequences separated by nonviral sequences (data not shown).

Thus, it appears that H1 was a "jackpot" cell in respect to the uptake and integration of many fragments of viral DNA. Many of the viral inserts were biologically inactive. To search for the active Py insert(s) responsible for the transformed phenotype of H1, a secondary transfection of rat cells was performed by using total cellular H1 DNA. Analysis of the DNA from a colony of transformed rat cells (H2) resulting from this transfection revealed a single insert of viral DNA (Fig. 2). Restriction enzyme analysis of H2 DNA with enzymes that cut Py DNA at multiple sites (*Sac* I, *Pvu* II, and *Msp* I) revealed that an intact early region was present and that continuous viral sequences existed from the *Hae* II site at Nt 95 (see below) up to the *Sac* I site at Nt 4,341 (compare cleavage of H2 and Py DNA in Fig. 2*C*). The H2 cells synthesized the Py large (M_r 100,000), middle (M_r 56,000), and small (M_r 22,000) tumor antigens and like H1 cells were highly tumorigenic in syngeneic Fischer rats.

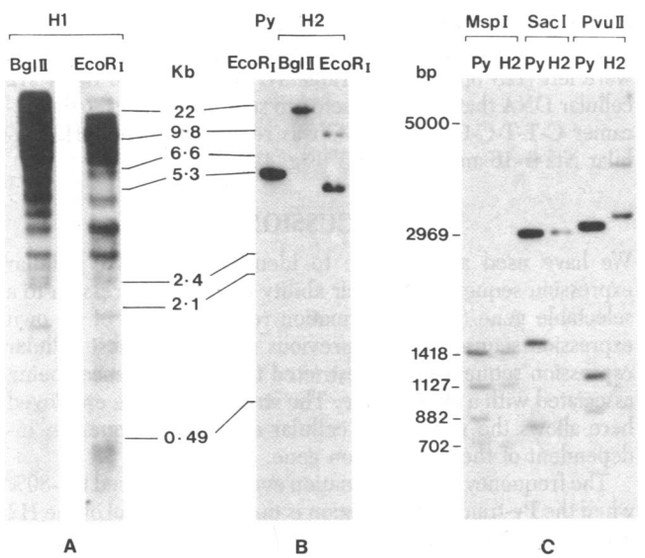

FIG. 2. Analysis of Py DNA sequences in H1 and H2 cellular DNA. Ten micrograms of H1 or H2 cellular DNA was digested with the indicated restriction endonucleases. The resulting DNA fragments were fractionated by electrophoresis in 1% agarose gels (*A* and *B*) and in 1.6% agarose (*C*), transferred onto nitrocellulose paper, and hybridized with ^{32}P-labeled Py probe as described (26, 27). The lanes (Py) contain 50 pg of Py DNA cleaved with the indicated enzymes. The gels were calibrated by *Hin*dIII digest of λDNA.

As the expression of the viral early region in H2 cells results from the influence of upstream 5′ sequences (see below), it was of interest to determine where the 5′ end of viral mRNAs was being initiated. The 5′ ends of the H2 Py early mRNAs were mapped by using the nuclease S1 procedure utilized by Kamen *et al.* (7, 28, 29) and were found to lie in the same region of the viral DNA as bona fide Py early region RNAs (Nts 148–152) (Fig. 3).

Transforming Activity and Sequence Determination of Cloned H2 Host Expression Sequences. The viral DNA and adjacent host sequences present in H2 cellular DNA were cloned

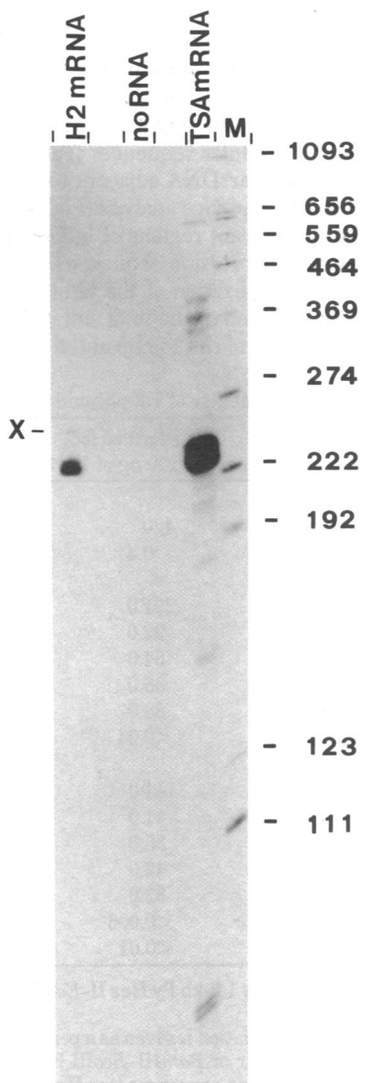

FIG. 3. Mapping of 5′ end of Py early mRNAs in H2 cells. The 5′ ends of poly(A)$^+$ mRNAs in H2-transformed rat cells were localized by hybridization to the Py *Hin*fI DNA probe and by fractionation of nuclease S1-resistant products on an 8% polyacrylamide gel. The quantities of RNA used were: H2 mRNA, 20 μg; and Py temperature-sensitive *A* mutant (TSA) shift-up, polyadenylylated cytoplasmic mRNA, 0.2 μg (7). Lane M contains 5′ ^{32}P-labeled *Dde* I fragments of Py DNA as size markers (shown in bp). The band designated X is an artefact as a result of incomplete nuclease S1 digestion, as detected by Kamen *et al.* (7).

by using λgt.WESλB from partial libraries of fractionated *Eco*RI-cleaved total cellular DNA (31). Consistent with the blot hybridization data (Fig. 2), two *Eco*RI fragments, 9 and 4.1 kb, containing Py sequences were cloned. The 9-kb fragment contained 1.5 kb of the 5′ end of the Py early region (transforming region) and 7.5 kb of cellular DNA, whereas the 4.1-kb fragment contained 2.8 kb of Py DNA (3′ ends of early and late regions) and 1.3 kb of cellular DNA (Fig. 1). The 9-kb *Eco*RI fragment (pL) and *Bam*HI–*Eco*RI subfragments containing the 1.5-kb viral-transforming region and either 149 bp (pB1), 1,120 bp (pB2), or 1,520 bp (pB3) of 5′ adjacent cellular sequences (Fig. 1) were subcloned into the plasmid pAT153 and tested for their transforming ability. No detectable differences in transforming activity were noted between the four plasmids containing different amounts of H2 cellular sequences (Table 1). In numerous experiments the transforming ability of these plasmids was consistently lower (20–40%) than that of the plasmid harboring the Py *Bam*HI–*Eco*RI fragment (that contains the Py expression sequences and transforming region) (Table 1) (Fig. 1). However, the transforming frequency of the four plasmids was still at least 100-fold higher than that of a plasmid containing the Py *Hae* II–*Eco*RI fragment (intact transforming region) lacking any H2 cellular sequences (Table 1).

The 149-bp H2 cellular DNA adjacent to the viral DNA in pB1 was subjected to sequence analysis (Fig. 4). This sequence did not show any significant regions of homology with the absent Py upstream 5′ expression sequences. As expected, the *Hae* II site used for the ligation of the viral and cellular DNA was present at the junction of the viral and cellular sequences. To determine how much of the 149 bp of the cellular sequences

Table 1. Transforming ability of H2 plasmids

Plasmid DNA	Transformed foci, no. per 100 ng of DNA*	% efficiency†
Group 1‡		
pPy *Bam*HI linear	420	100.0
pPy *Hae* II–*Bam*HI	0.4	0.1
Group 2§		
pPy *Bam*HI–*Eco*RI	291.0	100.0
pL	92.0	31.6
pB3	84.0	28.9
pB2	86.0	29.6
pB1	88.0	30.2
pPy *Hae* II–*Eco*RI	<0.04	
Group 3¶		
pPy *Bam*HI–*Eco*RI	144.0	100.0
pB1	41.0	28.5
pBΔ17	35.0	24.3
pBΔ18	42.0	29.1
pBΔ24	32.0	22.2
pBΔ20	<0.006	
pPy *Hae* II–*Eco*RI	<0.01	

* Measured per 100 ng of the 1.5-kb Py *Hae* II–*Eco*RI-transforming region.

† The efficiency of transformation is given as a percentage of a Py fragment (either *Bam*HI linear or *Bam*HI–*Eco*RI fragment) containing transforming and expression sequences (see Fig. 1).

‡ Comparison of transforming efficiencies of plasmids containing the Py *Bam*HI linear and the Py *Hae* II–*Bam*HI DNA fragment (see Fig. 1).

§ Comparison of the transforming efficiencies of plasmids containing the Py *Bam*HI–*Eco*RI and Py *Hae* II–*Eco*RI fragments and plasmids containing the Py *Hae* II–*Eco*RI fragment and various amounts of H2 cellular DNA (pL, pB3, pB2, and pB1) (see Fig. 1).

¶ Comparison of the transforming efficiencies of plasmids containing the Py *Bam*HI–*Eco*RI and Py *Hae* II–*Eco*RI fragments and plasmid pB1 and deletions of pB1 generated by BAL-31 (pBΔ17, pBΔ18, pBΔ24, and pBΔ20) (see Fig. 4).

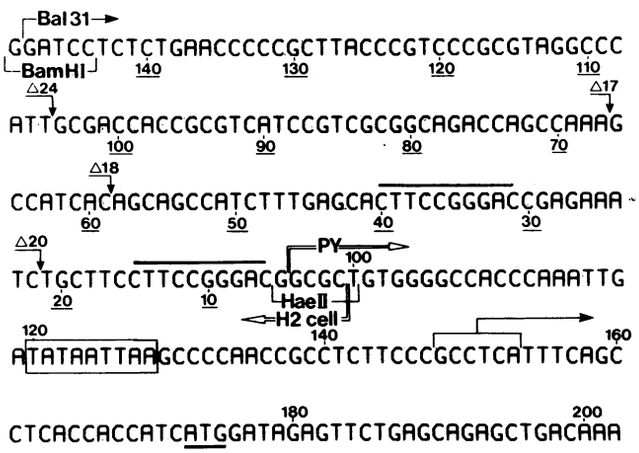

FIG. 4. Sequence of H2 cellular and Py DNA in plasmid pB1 and BAL-31 deletions pBΔ17, pBΔ18, pBΔ20, and pBΔ24. The sequence of H2 cellular DNA and part of the adjacent Py DNA in the plasmid pB1 is shown. The 149 bp of H2 cellular sequences are numbered (beneath sequence) from the *Hae* II site toward the *Bam*HI site. The numbering of the Py sequence (above sequence) is that of Soeda *et al.* (38). The nucleotides of the *Hae* II site are common to both sequences. The Py TATA sequence (Py Nts 120–128) is boxed, the region of initiation of early transcription (Py Nts 148–153) is overlined with an arrow, and the ATG used for the initiation of the Py early proteins (Py Nts 173–175) is underlined. BAL-31 digestion from the *Bam*HI site was used to generate the deletion derivatives pBΔ17, pBΔ18, pBΔ20, and pBΔ24. These derivatives contain the *Xho* I linker C-C-T-C-G-A-G-G at H2 cellular Nt 21 (pBΔ20), Nt 66 (pBΔ17), Nt 58 (pBΔ18), and Nt 104 (pBΔ24). The decamer C-T-T-C-C-G-G-G-A-C found twice at H2 cellular Nts 6–15 and Nts 30–41 is overlined.

were required for expression of the viral-transforming region, various amounts of H2 cellular sequences were deleted inward from the *Bam*HI site by using BAL-31 exonuclease (34) and the extent of the deletions was determined by DNA sequence analysis (Fig. 4). It was observed that there was no loss in transforming activity (pBΔ18) when up to 58 bp of the cellular sequences were present (91 bp deleted), whereas the transforming activity was lost (pBΔ20) when only 21 bp of host sequences were left (128 bp deleted) (Table 1). Within the 58 bp of H2 cellular DNA that were subjected to sequence analysis, the decamer C-T-T-C-C-G-G-G-A-C was repeated twice at H2 cellular Nts 6–16 and Nts 31–41 (Fig. 4).

DISCUSSION

We have used a procedure to identify and isolate cellular expression sequences by their ability to confer expression to a selectable gene (Py transformation region) devoid of its own expression sequences. The previous isolation of most cellular expression sequences was restricted to those sequences being associated with a cloned gene. The strategy we have employed here allows the isolation of cellular expression sequences independent of their companion gene.

The frequency of transformation events is decreased 60–80% when the Py-transforming region is under the control of the H2 cellular sequences in comparison to the Py expression sequences. This might indicate a difference in the strength of the viral and cellular expression sequences. On the other hand, there are no distinguishable differences in either the size, morphology, or time of appearance of the transformed colonies, induced either as foci on plastic or colonies in agar, by either the H2 cellular or Py expression sequences controlling the Py-transforming region. Thus, the difference in transformation frequencies might be explained if the chromosomal location of

particular integrations preferentially inhibited the functioning of the H2 cellular expression sequences in respect to the Py expression sequences.

It is not yet clear whether elements of the cellular H2 sequences play a role in the promotion or enhancement of transcription. The finding that in H2 cells initiation of Py early-region transcription occurs at the same nucleotides as in complete Py DNA (7) may reflect only the influence of the single TATA sequence present in the region. From the BAL-31 deletion mapping it appears that the H2 sequences important for expression lie within the first 58 bp. This would place these elements between −50 and −108 bp from the initiation of transcription. In other genes the CAAT sequence thought to be associated with expression is usually found at about 80 bp (−80) from the start of transcription (8–10). No H2 sequences are observed that correlate well with the canonical CAAT sequence (9), but a poor sequence fit may occur at H2 cellular Nts 29–21 (−71 to −79) (G-A-G-A-A-A-T-C-T). A striking feature within the first 58 H2 bp is the repeat of the decamer C-T-T-C-C-G-G-G-A-C at Nts 6–15 and 31–40 (Fig. 4).

Exactly what role these sequences play in expression awaits further deletion mapping and mutagenic studies of the H2 cellular sequences. A computer analysis (35) of upstream 5′ sequences of a number of eukaryotic cellular and viral genes has not shown any striking homologies with the H2 cellular sequence. Experiments on the influence of position and orientation of the H2 cellular sequence in relation to a structural gene may distinguish between promoter and enhancer activity. From blot hybridization studies, it is clear that the 149 bp of the H2 cellular DNA that was subjected to sequence analysis does not represent either high or middle repetitive sequences in rat or mouse DNA (unpublished data). Further isolation of other cellular sequences, by using procedures similar to those described here, should allow a repertoire of expression sequences to be identified which may help our understanding of gene expression.

We thank Earl Ruley, Adrian Hayday, Sharon Boast, and Delli Bovi for their help and discussion at various stages of this work and John Wyke, Malcolm Parker, Mike Waterfield, and Nancy Hogg for their useful suggestions in the preparation of this manuscript. Part of this work was supported by the Consiglio Nazionale delle Ricerche and the Ministero Della Publica Istruzione of Italy.

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