MOLECULAR AND CELLULAR BIOLOGY, Nov. 1995, p. 6246–6255 Vol. 15, No. 11
0270-7306/95/$04.00+0

Adenovirus Type 12-Induced Fragility of the Human *RNU2* Locus Requires U2 Small Nuclear RNA Transcriptional Regulatory Elements

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Received 3 May 1995/Returned for modification 12 July 1995/Accepted 26 July 1995

Infection of human cells with oncogenic adenovirus type 12 (Ad12) induces four specific chromosome fragile sites. Remarkably, three of these sites appear to colocalize with tandem arrays of genes encoding small, abundant, ubiquitously expressed structural RNAs—the *RNU1* locus encoding U1 small nuclear RNA (snRNA), the *RNU2* locus encoding U2 snRNA, and the *RN5S* locus encoding 5S rRNA. Recently, an artificial tandem array of the natural 5.8-kb U2 repeat unit has been shown to generate a new Ad12-inducible fragile site (Y.-P. Li, R. Tomanin, J. R. Smiley, and S. Bacchetti, Mol. Cell. Biol. 13:6064–6070, 1993), demonstrating that the U2 repeat unit alone is sufficient for virally induced fragility. To identify elements within the U2 repeat unit that are required for virally induced fragility, we generated cell lines containing artificial tandem arrays of the entire 5.8-kb repeat unit, an 834-bp fragment spanning the U2 gene alone, or the same 834-bp fragment from which key U2 transcriptional regulatory elements had been deleted. The U2 snRNA coding regions within each artificial array were marked by an innocuous single base change (U to C at position 87) so that the relative expression of supernumerary and endogenous U2 genes could be monitored by a primer extension assay. We find that artificial arrays of both the 5.8- and the 0.8-kb U2 repeat units are fragile but that arrays lacking either the distal sequence element or both the distal and the proximal sequence elements of the promoter are not. Surprisingly, variations in repeat copy number and/or transcriptional activity of the artificial arrays do not appear to correlate with the degree of Ad12-inducible fragility. We conclude that U2 transcriptional regulatory elements are required for virally induced fragility but not necessarily U2 snRNA transcription per se.

A number of cancers (60) as well as several kinds of heritable mental retardation known as fragile X syndrome (16, 18) are caused by or associated with chromosome fragile sites. Fragile sites typically appear in metaphase spreads as nonstaining or heterochromatic gaps, often accompanied by a dislocation in the chromosome axis. Although clinically observed fragile sites seldom reflect frank chromosome breakage, fragile sites colocalize with over 60% of recurrent cancer chromosome breakpoints (59). This suggests that oncogenic rearrangements and translocations are often caused by recombination at fragile sites or by breakage and subsequent chromosome fusion (6, 16, 18, 41, 59, 60). Fragile sites can be induced by a variety of methods, including abnormal growth conditions, drug or chemical treatment, and viral infection; for this reason, fragile sites are usually classified according to the method and efficiency of induction (17, 49). Although there are likely to be many different causes of chromosome fragility, it has been shown in at least one case that a fragile site can correspond to a region of incompletely condensed chromatin (12). A defect in chromatin condensation could explain why fragile sites are physically weak and genetically recombinogenic and why certain fragile sites serve as preferential targets for viral integration (references 39, 46, and 52, but see also reference 57). Fragile sites are also conserved among primates (45), and a majority of the breakpoints observed in the evolution of primate chromosomes fall at or near known fragile sites (33).

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Infection of human cells with the nononcogenic adenovirus type 2 or 5 (Ad2 or Ad5) induces generalized chromosome fragility (6), but infection with the oncogenic Ad12 induces four, and only four, specific chromosome fragile sites (63). These four Ad12-induced fragile sites have been mapped, in order of decreasing fragility, to 17q21-22, 1p36, 1q21, and 1q42-43 (30, 31, 63). Since these four sites are not preferential targets for adenoviral integration (reference 32, but see reference 52), virally inducible fragility must be an intrinsic property of the underlying chromosomal regions, perhaps reflecting some unusual aspect of chromatin structure, gene organization, transcription, or DNA replication. Remarkably, three of the four sites appear to colocalize with large tandem arrays of genes encoding small, abundant, ubiquitously expressed structural RNAs—the *RNU1* locus encoding U1 small nuclear RNA (snRNA) at 1p36 (23, 35), the *RNU2* locus encoding U2 snRNA at 17q21-22 (14, 22), and the *RN5S* locus encoding 5S rRNA at 1q42-43 (47, 48). The fourth fragile site colocalizes with the *PSU1* locus at 1q21 (23), an old and highly divergent family of U1 snRNA genes that are no longer expressed as stable U1 snRNA (24) but which may still retain active transcriptional regulatory elements.

Colocalization of Ad12-inducible fragile sites with active gene clusters suggested that the gene clusters themselves were required, and might be sufficient, for virally induced fragility (22). Subsequent work on the U2 genes has confirmed this idea. The human U2 genes are organized as a single, essentially perfect tandem array containing 5 to 25 copies of a 5,834-bp repeat unit at 17q21-22 (14, 22, 37, 50, 55); the U2 array therefore spans 30 to 150 kb. Durnam et al. demonstrated by classical in situ hybridization with ^{3}H-labeled U2 probes that the Ad12-induced fragile site at 17q21-22 was not only near, but occasionally fell within, the *RNU2* locus, as evidenced by

splitting of the U2 hybridization signal (11). This result more strongly implicated the U2 array itself as the cause of virally induced fragility, but it was also possible that unraveling of the U2 array was a consequence rather than the cause of fragility. More recently, Li et al. have generated cell lines containing stably integrated, artificial arrays of the full 5.8-kb U2 repeat unit which exhibit the same Ad12-induced fragility as the natural *RNU2* locus (21). This proves that the U2 repeat unit alone is sufficient for virally induced fragility and that fragility does not depend on the immediate flanking sequences of the U2 tandem array or on the chromosomal microenvironment surrounding the natural *RNU2* locus.

The ability of Ad12 to induce specific chromosome fragile sites may be due solely to the E1 region of the virus; indeed, constitutive fragility can be observed at 17q21-22 in hybrid mouse-human cell lines stably transfected with the Ad12 E1 region alone (12). The E1 region includes two transcription units: E1A generates three related, multifunctional proteins by alternative 5′ splicing, and E1B generates 19,000- and 55,000-molecular-weight proteins (19K and 55K proteins) from a single mRNA with overlapping reading frames. Virally induced fragility is unaffected by mutations in the E1B 19K protein (12, 41) but is 70% reduced by a mutation in the 55K protein (41). In addition, a hybrid virus (T1227) carrying the first exon of Ad12 E1A in an Ad5 background was unable to induce chromosomal damage. Thus, the Ad12 E1B 55K protein may be sufficient for virally induced fragility, although a role for one of the E1A proteins has not been rigorously excluded.

Ad12-induced chromosome fragility requires an interaction between viral functions and the intrinsic properties of specific chromosomal sites. To understand the causes of virally induced fragility at the molecular level, we have begun by identifying the minimal elements within the U2 repeat unit required for Ad12-induced fragility. We have generated cell lines containing tandem arrays of the intact 5.8-kb U2 repeat unit, an 834-bp U2 minigene, and U2 minigenes from which key transcriptional regulatory elements have been deleted. In each case, the U2 snRNA coding regions were marked by a harmless single base change (U to C at position 87 [U87C]) that enabled us to monitor the relative expression of supernumerary and endogenous U2 genes by a simple primer extension assay. We found that artificial arrays containing the complete U2 transcription unit are fragile but that arrays lacking the enhancer-like distal sequence element (DSE) (2, 3, 27) are not. The degree of Ad12-inducible fragility did not correlate, however, with variations in repeat copy number and/or transcriptional activity. Thus, virally induced fragility requires U2 transcriptional regulatory elements but not necessarily U2 snRNA transcription per se. These results provide the groundwork for a detailed molecular understanding of the chromosomal elements, as well as the viral and host factors, required for the site-specific chromosomal fragility induced by Ad12. Gargano et al. (13a) have reached very similar conclusions.

MATERIALS AND METHODS

Clone construction. To generate the marked U2 minigene, an 824-bp fragment extending from the *Stu*I site upstream of the U2 coding region to the *Hin*fI site just downstream of the 3′-end formation signal (3, 37) was subcloned into M13mp18. The U87C mutation within the 188-bp U2 coding region was introduced by standard techniques (19) and verified by sequence analysis. The marked U2-U87C minigene was excised as an *Eco*RI-to-*Bam*HI fragment and transferred to pUC18, and a *Bgl*II linker (New England Biolabs) was inserted into the filled-in *Eco*RI site. This clone was designated mU2, where m indicates a minigene. To generate a marked, intact 5.8-kb U2 repeat unit, the *Hin*cII-to-*Apa*LI fragment of mU2 was used to replace the corresponding sequence in the plasmid pTPK18Eco (protocol available upon request). The parent construct, pTPK18, contains a 5.8-kb *Kpn*I fragment spanning the entire U2 repeat unit (37); the unique *Xmn*I site within the U2 repeat unit was destroyed by insertion of an *Eco*RI linker to generate pTPK18Eco. The resulting clone was designated iU2, where i indicates an intact gene (Fig. 1A).

Transcriptional regulatory elements were deleted from mU2 (Fig. 1A). The DSE was excised by digestion with *Hin*cII and *Sfu*I; the *Sfu*I end was then filled in, and the plasmid was religated to generate mU2ΔDSE. The DSE and proximal sequence element (PSE) were deleted from *Hin*cII to *Fok*I by ligating two fragments of mU2. mU2 was first digested with *Fok*I, and the ends were filled in. The blunt *Fok*I-to-*Bam*HI fragment spanning the marked U2 gene was then excised with *Bam*HI and ligated to the *Bam*HI-to-*Hin*cII vector fragment of mU2 spanning sequences upstream of *Hin*cII as well as all of pUC18. This clone was designated mU2ΔDSEΔPSE. The deletions in mU2ΔDSE and mU2ΔDSEΔPSE were confirmed by sequence analysis. An additional clone, designated mU2+CT, contained the U2 minigene along with the $(CT)_{70}$ dinucleotide repeat located immediately downstream from the U2 transcription unit (Fig. 1A and reference 27). mU2+CT was generated by ligating the filled-in *Rsr*II-to-*Stu*I fragment from iU2 into the filled-in *Xma*I site of pUC18Bgl (a modified pUC18 in which the polylinker *Eco*RI site has been destroyed by insertion of a *Bgl*II linker).

The selection cassette was composed of two parts (Fig. 1A). The neomycin resistance gene was excised from pMC1NEO (Stratagene) as an *Xho*I-to-*Bam*HI fragment, filled in, and cloned into the *Sma*I site of pUC18Bgl. A mouse dihydrofolate reductase (DHFR) gene carrying a point mutation that renders it resistant to high levels of methotrexate (43) was then cloned into the *Kpn*I site to generate plasmid E1. To reduce the risk of promoter occlusion or transcriptional interference, all four orientations of the two genes (head-to-head, tail-to-tail, and head-to-tail) were made and tested as monomeric plasmids for the relative frequency of Geneticin-resistant colonies. The E1 selection cassette with the Neo^r gene upstream of the DHFR gene (Fig. 1A) yielded a 10- to 20-fold-higher frequency of resistance than the other orientations (DHFR gene upstream of the Neo^r gene, convergent or divergent transcription units).

Cloning and analysis of cell lines. Generation of cell lines that contain large tandem U2 arrays was not straightforward; large tandem arrays generated by in vitro ligation are easily sheared during standard experimental manipulations, and smaller contaminating arrays are preferentially incorporated during transfection. We therefore conducted numerous pilot experiments as summarized here. Our goal was to generate large head-to-tail tandem U2 arrays doped by an occasional E1 selection cassette; we sought to avoid generating any oligomeric products containing the E1 cassette, as these would be preferentially incorporated upon transfection and would therefore require characterization of many more cell lines. Tandem arrays were constructed by ligation of fragments with dissimilar but compatible *Bam*HI and *Bgl*II ends; the desired head-to-tail ligations generate hybrid *Bgl*II-*Bam*HI restriction sites that are resistant to digestion by either enzyme, while undesirable head-to-head and tail-to-tail ligations regenerate *Bgl*II and *Bam*HI sites which can be recycled by digestion with both enzymes. To reduce the risk of damaged ends which act as chain terminators during ligation, *Bgl*II-to-*Bam*HI fragments of the U2 constructs and the E1 selection cassette (Fig. 1A) were purified from low-melting-point agarose (13) just before use. Using T4 DNA ligase, we performed a series of ligations in which the total fragment concentration was varied from 5 to 500 μg/ml and the ratio of U2 to E1 fragments was varied from 10:1 to 100:1. The ligation products were characterized, both before and after digestion with *Bgl*II and *Bam*HI, by field inversion gel electrophoresis (FIGE), ethidium bromide staining, and blotting. Under the FIGE conditions used, monomers through decamers were resolved as ladder bands, while larger multimers ran as a smear. Although we expected oligomeric products at low DNA concentrations, we were surprised to find significant amounts of oligomeric products (circularized monomers and dimers, etc.) even at the highest DNA concentrations tested. We were unable to reduce the yield of oligomeric products, or to generate larger tandem arrays, by simultaneous ligation and digestion with *Bgl*II plus *Bam*HI or by alternating rounds of ligation and digestion. This may indicate that most attempts to increase ligation are effectively opposed by chain termination due to exonuclease, phosphatase, and/or mechanical shear. In fact, ligation at high DNA concentrations generated solutions of high viscosity that were obviously subject to mechanical shear even when handled with widebore pipette tips. Ligation in the presence of 50 μM spermidine reduced the viscosity of the ligation reaction mixture and, at least in our hands, generated larger arrays. This is consistent with reports that polyamines condense DNA, leading to more compact, rigid molecules with reduced viscosity and increased resistance to shear (5). As expected, a U2-to-E1 ratio of 100:1 reduced incorporation of the selection cassette into oligomeric products, and we also found, by varying the temperature from 4 to 37°C, that residual monomer was minimized by ligation at 25°C. Finally, we attempted to enrich for larger tandem arrays by preparative FIGE but found that the arrays could not be recovered from agarose without significant shearing.

In our final protocol for generating tandem arrays, we used a 100:1 ratio of U2 to E1 and ligated 25 μg of DNA fragments at 500 μg/ml for 3 to 4 h at 25°C. The reaction mixture was then diluted twofold to reduce the viscosity, keeping the spermidine at 50 μM, and digested with a twofold excess of *Bgl*II and *Bam*HI to eliminate head-to-head and tail-to-tail junctions. The DNA was then precipitated with ethanol, washed with 70% ethanol, and gently resuspended in 250 μl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) containing 50 μM spermidine.

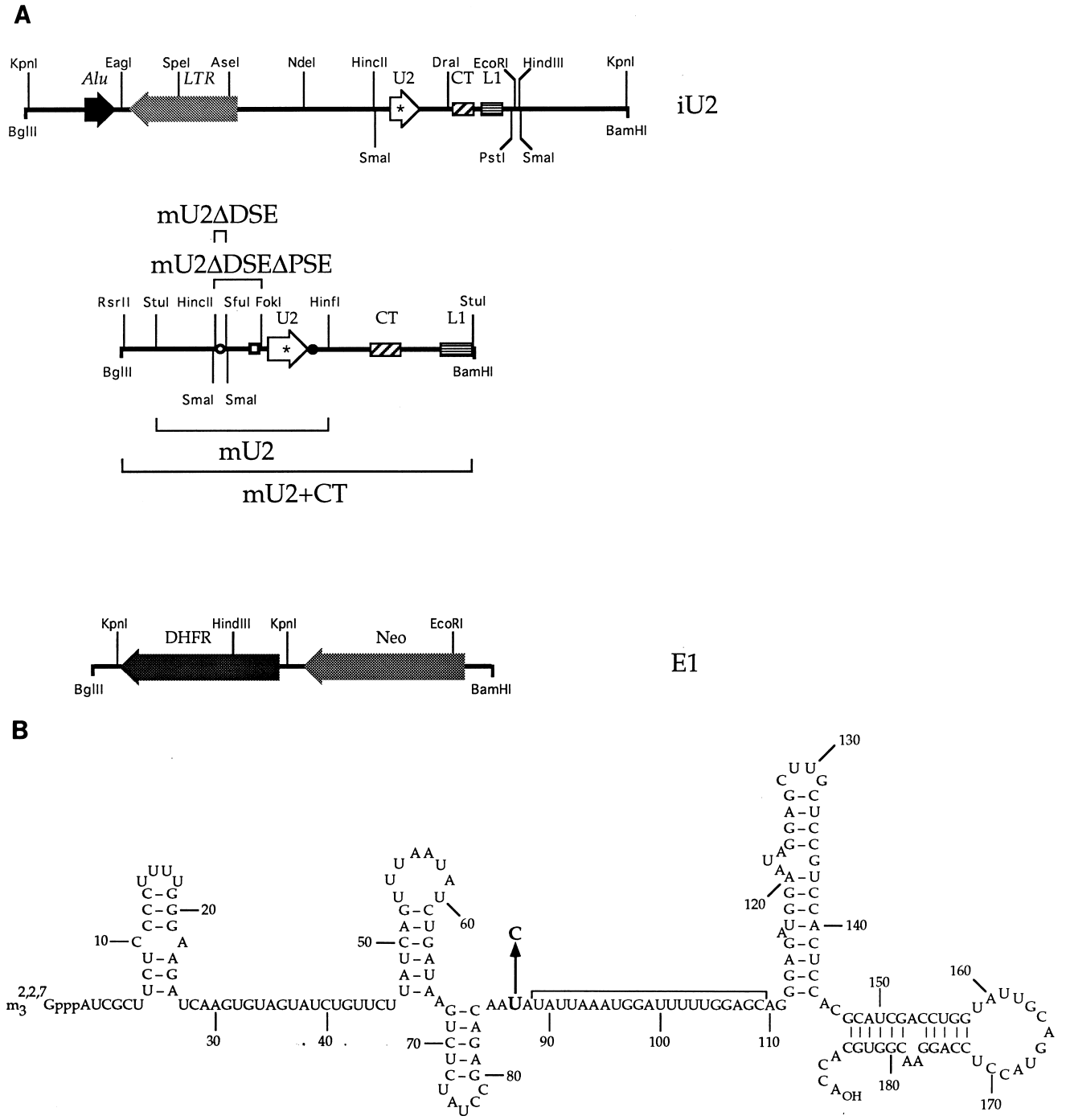


FIG. 1. (A) Constructs used to generate artificial U2 tandem arrays. All U2 genes contain the U87C mutation (*) to distinguish U2 snRNAs transcribed from the endogenous and artificial arrays (also see panel B). iU2 (for intact) is a full-length 5.8-kb U2 repeat unit (top panel) in which the unique *Xmn*I site has been destroyed by insertion of an *Eco*RI linker. Artificial arrays of iU2 can be distinguished from endogenous U2 arrays which are not cut by *Eco*RI (37, 50). Only the diagnostic *Sma*I and *Pst*I sites (see Fig. 2) are shown below the gene. Also indicated are the location of a solo long terminal repeat (LTR), the CT dinucleotide repeat, and a 5′-truncated L1 sequence (37). mU2 (for mini) is a U2 minigene (middle panel); mU2+CT includes the downstream $(CT)_{70}$ dinucleotide repeat as well. The U2 coding region (open arrow) is preceded by two transcriptional regulatory elements known as the DSE and PSE (open circle and square, respectively) and is followed by the 3′-end formation signal (filled circle). The extent of mU2 and mU2+CT (brackets below the map) and the regions deleted from mU2 to generate mU2ΔDSE and mU2ΔDSEΔPSE (brackets above the map) are indicated. The E1 selection cassette (bottom panel) is also shown. Note that all constructs have dissimilar *Bgl*II and *Bam*HI ends to allow head-to-tail ligation (see text). (B) U2 snRNA sequence and accepted secondary structure (61). The U87C mutation (arrow) was chosen because this position is phylogenetically variable and adjacent to a long single-stranded region, which should facilitate annealing with a primer complementary to U2 positions 89 to 109 (bracket).

We compared transfection by Ca_2PO_4 precipitation and electroporation using in vitro-ligated tandem arrays prepared as described above. The recipient cells were the pseudodiploid fibrosarcoma line HT-1080 (gift from S. Bacchetti [21]). Ca_2PO_4 precipitation and electroporation generated Geneticin-resistant colonies with comparable efficiencies when the E1 selection cassette was used alone, but Ca_2PO_4 precipitation was roughly twice as efficient as electroporation for transfection of large tandem arrays and generates larger arrays on average. We suspect that Ca_2PO_4 precipitation protects large DNA molecules from mechanical shear, while small DNA molecules preferentially traverse transient membrane pores during electroporation. Ultimately, all cell lines were generated by Ca_2PO_4 precipitation (Gibco/BRL) except cell line mU2 C12 (electroporated in a 0.4-cm-diameter cuvette at 0.2 kV and 960 μF with a Bio-Rad Gene Pulser with a Capacitance Extender). HT-1080 cells were grown in minimal essential medium (Gibco/BRL) with 10% fetal bovine serum, the cultures were split 24 h before transfection to give 10^6 cells per 100-mm-diameter plate, and the medium was replaced 1 h before transfection. The medium was changed again 18 h after transfection, and 24 h later, the cells were divided among 10 to 12 150-mm-diameter plates with 25 ml of media containing 600 μl of a 25-mg/ml stock of Geneticin (Gibco/BRL). The medium was changed every 3 to 4 days over a 14- to 17-day period. The resulting colonies were collared with a cloning cylinder, trypsinized, and transferred to 48-well microtiter plates. Individual clonal cell lines were grown until frozen stocks and genomic DNA plugs could be made.

Plugs were made by resuspending trypsinized cells from a 60-mm-diameter plate in 300 μl of phosphate-buffered saline. To this suspension, 300 μl of 1% Incert Agarose (FMC Corp.) at 40°C was added, the suspension was mixed, and solution was pipetted into the plug mold (Medical Specialties, Baltimore, Md.). Processing of the plugs and FIGE were performed as described previously (37). "Unblots" of the FIGE gels are modified genomic blots in which the dried agarose gel is used directly instead of a transfer of the DNA to nitrocellulose or nylon (28).

Relative expression of marked U2 genes. Cells were collected by trypsinization from nearly confluent 100-mm-diameter plates and lysed with Nonidet P-40 as described elsewhere (62), and total RNA was prepared by phenol extraction. Primer extension analysis of total RNA was as described previously (62) except that ddATP was used in place of dATP and extension products were resolved on a 15% sequencing gel. Phosphorimager analysis was performed with a GS-250 Molecular Imager (Bio-Rad) and the program Molecular Analyst 2.0.

Ad12-induced fragility. Cells were infected with Ad12 (strain Huie) at a multiplicity of infection of 10 (11, 21). About 17 h after infection, cells were treated with colcemid at 0.1 μg/ml for 3 h, and metaphase spreads were then prepared by standard techniques of hypotonic shock and methanol-acetic acid fixation (4).

FISH. Natural and artificial U2 loci were labeled for fluorescent in situ hybridization (FISH) by using biotinylated probes. Briefly, slides were denatured in a solution of 70% formamide and 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 70°C for 2 min and then subjected to immediate serial dehydration in ice-cold 70, 90, and 100% ethanol. The slides were then air dried at room temperature. Hybridization was performed in a solution containing 50% formamide, 2× SSC, 10% dextran sulfate, 500 μg of salmon sperm DNA per ml, and 50 μg of biotinylated DNA probe per ml. After 5 min of denaturation at 70°C, 20 μl of hybridization solution was applied to each slide, and the slides were then covered with coverslips and sealed with rubber cement. Hybridization was performed overnight in a moist chamber at 37°C. The coverslips were then removed, and the slides were washed twice in a solution of 50% formamide and 2× SSC at 42°C for 10 min and twice in 0.1× SSC at 37°C for 5 min. The washed slides were incubated with 5 μg of fluorescein-conjugated avidin DCS per ml in a solution containing 4× SSC, 3% bovine serum albumin, and 0.1% Tween 20 for 30 min. After three washes in a mixture of 4× SSC and 0.1% Tween 20 for 10 min, the slides were counterstained with DAPI (4,6-diamidino-2-phenylindole; 200 ng/ml) and mounted in a solution containing 90% glycerol, 0.1 M Tris-HCl (pH 8.0), and 2.3% DABCO antifade (1,4-diazabicyclo-2,2,2-octane).

Images were collected with a Zeiss Axioskop epifluorescence microscope equipped with a cooled, charge-coupled device (CCD) camera (Photometrics) and controlled by a computer interface software package (CCD Image Capture). The source images were pseudocolored and merged by using computer software (GeneJoin) available through the Office of Cooperative Research, Yale University. The equipment and protocols have been described in detail elsewhere (4).

RESULTS

Construction of artificial U2 arrays. Li et al. previously demonstrated that an artificial tandem array of the natural 5.8-kb U2 repeat unit will generate a new Ad12-inducible fragile site (21). To better understand the causes of Ad12-induced fragility, we constructed cell lines containing artificial tandem arrays of five different U2 constructs (Fig. 1A). Tandem arrays were generated by using iU2, an intact 5.8-kb U2 repeat unit in which the *Xmn*I site has been destroyed by insertion of an *Eco*RI linker; mU2, an 824-bp minigene containing all essential transcriptional regulatory elements, the U2 coding region, and the 3′-end formation signal; and mU2+CT, an mU2 derivative including the downstream $(CT)_{70}$ dinucleotide tract. We also constructed two mU2 derivatives lacking key transcriptional regulatory elements: the DSE is deleted in mU2ΔDSE, and both the DSE and the PSE are deleted in mU2ΔDSEΔPSE. In each construct, the U2 gene contains a point mutation from U to C at position 87 in the U2 coding region (Fig. 1B). Although U2 snRNA is highly conserved in sequence and structure from yeasts to humans, position 87 is phylogenetically variable (61) and the U87C mutation is most unlikely to affect U2 snRNA expression or function. Use of the marked U2-U87C gene allowed us to monitor expression of the supernumerary U2 genes in the artificial arrays and to prove that these U2 snRNAs are functional, at least as judged by immunoprecipitation with anti-Sm antibodies (data not shown).

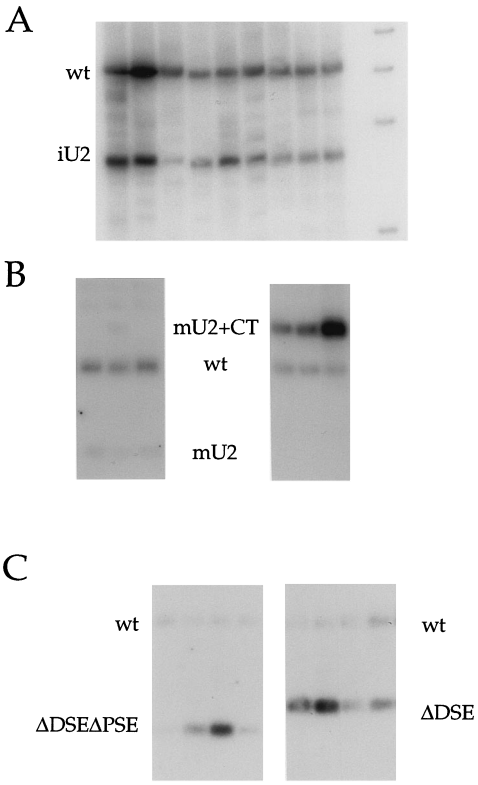


FIG. 2. Genomic organization of artificial U2 arrays. Agarose plugs were prepared from representative cell lines, digested as indicated below, and assayed by blotting with an mU2 probe (see Fig. 1A). (A) DNA from the iU2 cell lines, when digested with *Eco*RI and *Spe*I, yields a 3.4-kb fragment from the artificial iU2 tandem array and a 5.8-kb fragment from the wild-type (wt) resident *RNU2* loci. (B) DNA from the mU2 and mU2+CT cell lines, digested with *Sfu*I and *Pst*I, yields fragments of 824 (mU2), 1,702 (mU2+CT), and 1,356 (wild type) bp. (C) DNA from the mU2ΔDSE and mU2ΔDSEΔPSE cell lines, when digested with *Sma*I, yields fragments of 753 and 606 bp, respectively, compared with 1,469 bp for the wild type.

The intact U2 repeat unit (iU2). Geneticin-resistant cell lines were examined by genomic blotting for the presence of artificial U2 arrays. Initially, *Eco*RI digests were probed with the unique 1.9-kb *Spe*I-to-*Hin*cII fragment of the 5.8-kb U2 repeat (Fig. 1A). *Eco*RI is a "null cutter" which does not cut within the endogenous U2 arrays; the observed 5.8-kb band is therefore due solely to the artificial iU2 tandem array (Fig. 2A). The absence of 2.1- and 2.7-kb bands confirms that all

TABLE 1. Characteristics of cell lines containing artificial U2 tandem arrays

Cell line or RNA	Copy no. of marked U2 genes[a]	DNA ratio[b]	RNA ratio[c]	RNA/gene[d]	RNA/locus[e]	Fragility (%)[f]
iU2 A11	12	0.26	0.44	1.6	1.7	80–90
iU2 A24	25	0.56	0.91	1.6	3.5	10–20
iU2 A42	29	0.67	0.58	0.82	2.2	90–100
iU2 A36	30	0.68	0.52	0.71	2	80–90
iU2 A16	32	0.72	0.91	1.2	3.5	10–20
iU2 A37	48	1.1	1.8	1.6	7	90–100
iU2 A41	77	1.8	2.5	1.4	9.8	90–100
iU2 A40	79	1.8	0.58	0.31	4.4	90–100
mU2 C12	5	0.119	0.155	1.1	0.51	5–10
mU2 T38	6	0.128	0.142	0.9	0.46	5–10
mU2 L48	8	0.182	0.224	1.1	0.79	10–20
mU2ΔDSE 33	22	0.98	0.063	0.037	0.072	≤5
mU2ΔDSE 50	31	1.4	0.11	0.061	0.17	~10
mU2ΔDSE 34	53	2.4	0.062	0.015	0.07	<5
mU2ΔDSE 9	79	3.6	0.029	0.0005	0.0036	<5
mU2ΔDSE 25	112	5.1	0.039	0.0024	0.024	≤5
mU2ΔDSEΔPSE 14	8	0.36	0.027	0.0011	0.008	<1
mU2ΔDSEΔPSE 43	20	0.9	0.026	0	0	<1
mU2ΔDSEΔPSE 5	29	1.3	0.026	0	0	<1
mU2ΔDSEΔPSE 33	57	2.6	0.026	0.0023	0.024	<1
mU2ΔDSEΔPSE 48	78	3.5	0.023	0	0	<1
mU2ΔDSEΔPSE 42	98	4.4	0.033	0.0014	0.012	<1
HeLa RNA			0.011			
HT-1080 RNA			0.027			

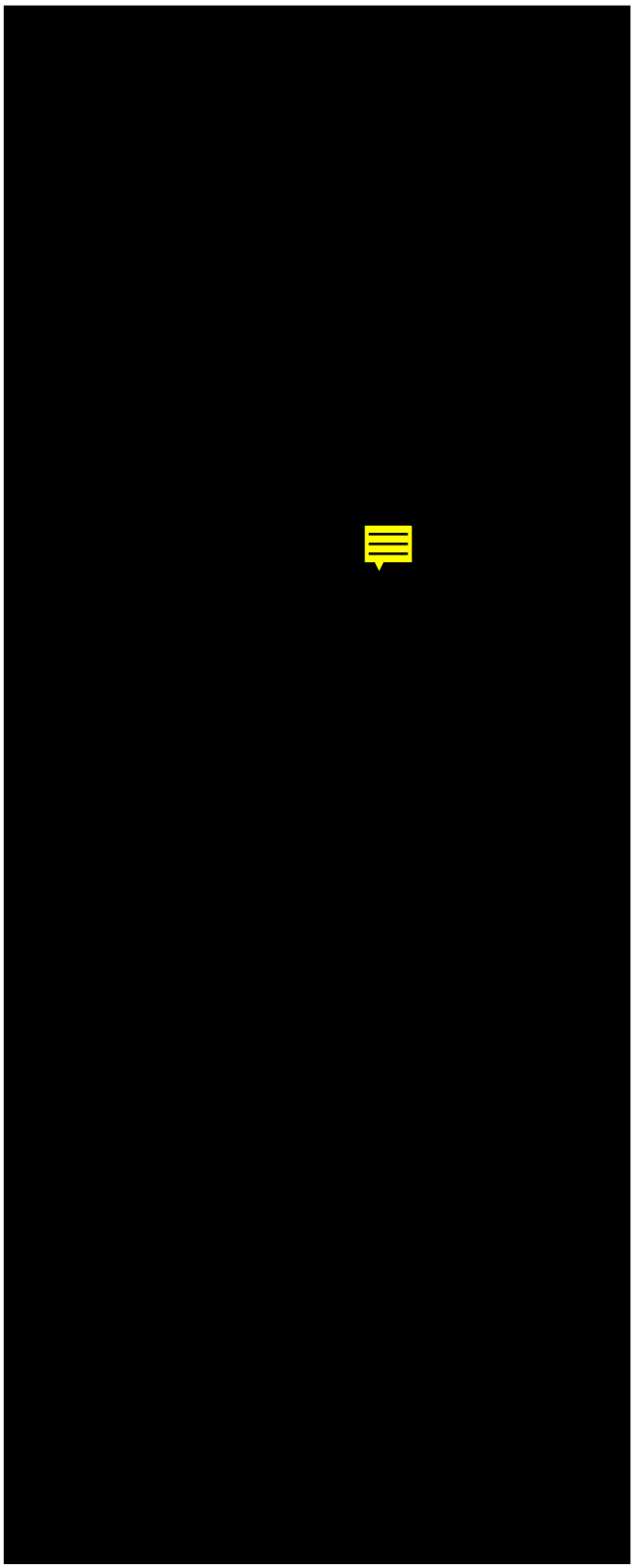
[a] Calculated from the DNA ratio and the known copy number of endogenous U2 genes (22 for pseudodiploid and 44 for pseudotetraploid HT-1080 cells [36]).
[b] Ratio of marked U2 to endogenous U2 signal as determined by phosphorimager analysis of genomic blots (see Fig. 2). Corrections were made for the extent of complementarity with the mU2 probe.
[c] Ratio of marked U2 snRNA to wild-type snRNA as determined by phosphorimager analysis of primer extension assays performed on total RNA (see Fig. 4).
[d] RNA ratio divided by the DNA ratio. This represents the relative expression of the marked U2 genes compared with that of the wild type.
[e] RNA ratio divided by the ratio of artificial U2 loci to endogenous *RNU2* loci. Assuming that all endogenous loci are equally expressed, this represents the relative expression per locus of the artificial and endogenous arrays.
[f] Relative frequency of breakage at artificial U2 loci compared with that at endogenous *RNU2* loci (see Fig. 3 for scoring of breaks); the range of fragility represents the variation observed in two separate experiments.

tandem repeats were head-to-tail, not head-to-head or tail-to-tail. Of 95 Geneticin-resistant cell lines screened, 25 had at least as many artificial iU2 as endogenous U2 repeats. These 25 cell lines were further characterized by digestion with *Bam*HI and *Bgl*II followed by FIGE. The gels were then dried and hybridized directly with the labeled probe (28). The number and size of artificial iU2 arrays were found to vary, and eight cell lines containing the largest unique iU2 arrays were selected for further study. To accurately measure the relative copy numbers of iU2 and U2 repeats in these eight lines, DNA was digested with *Eco*RI and *Spe*I; iU2 repeats are reduced to 3.4-kb fragments, while endogenous arrays yield monomeric 5.8-kb fragments (Fig. 2A). The ratio of the artificial to endogenous bands was determined by phosphorimager analysis, and the eight cell lines were found to contain 12 to 79 tandem copies of the 5.8-kb iU2 repeat unit (Table 1, DNA ratio).

We soon discovered, to our initial dismay, that all of the eight iU2 lines were tetraploid, apparently because tetraploid cells had almost overgrown the parental pseudodiploid HT-1080 cells. Tetraploidy is unlikely to be a response to transfection with supernumerary U2 genes, because pseudodiploid iU2 lines were also identified (Table 1). As ploidy was not expected to affect virally induced fragility, we continued to work with these lines although good metaphase spreads were more difficult to obtain.

The minigene construct (mU2). The drug-selected cell lines were first examined by genomic blotting with *Sfu*I, a "one cutter" for both the endogenous and the mU2 minigene repeat units. Blots were probed with the mU2 monomer, which detects both natural and artificial U2 genes, as well as the many 3′-truncated U2 retropseudogenes (51). Surprisingly, in three separate transfections, we found only seven cell lines that contained more than one of the 824-bp mU2 repeat units (1 of 31, 4 of 77, and 2 of 127 transfectants). Of these seven cell lines, only three exceeded the minimum array size required for detection by FISH, and none contained more than eight tandem repeats of mU2. The absence of tandem arrays containing more than eight copies of mU2 contrasts dramatically with the ease of obtaining large tandem arrays containing nearly 80 copies of the full 5.8-kb iU2 construct (Table 1). We do not know whether the copy number constraint reflects a problem with integration or stability of mU2 arrays but, as described in greater detail below (also see Table 1), the copy number constraint is relieved when U2 transcription is crippled by deletion of either the DSE (mU2ΔDSE) or both the DSE and the PSE (mU2ΔDSEΔPSE). Since both U1 snRNA (25) and U2 snRNA (3a) are subject to dosage compensation (i.e., snRNA levels are substantially independent of snRNA gene copy number), one interesting possibility is that large mU2 arrays might be unstable if mU2 is able to escape dosage compensation.

Attempts to characterize the three mU2 cell lines by digestion with *Bam*HI and *Bgl*II failed to give clear results, appar-



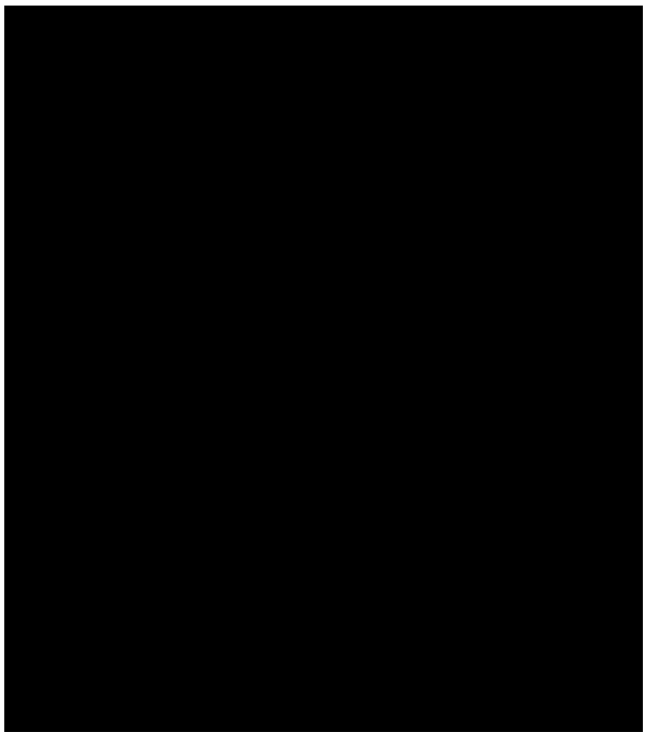


FIG. 3. Ad12-induced fragility of artificial U2 tandem arrays. (A) iU2 series. (B) mU2 series. (C) Cell line mU2ΔDSE 50. FITC, fluorescein isothiocyanate.

ently because abundant U2 retropseudogenes readily react with the minigene probe, but digestion with *Sfu*I and *Pst*I (Fig. 1A) resolved monomers of mU2 (824 bp) from endogenous U2 genes (1,356 bp) and from the smear of retropseudogenes above (Fig. 2B). The ratios of mU2 to endogenous U2 genes were then determined by phosphorimager analysis (Table 1).

Minigenes lacking transcriptional regulatory signals (mU2ΔDSE and mU2ΔDSEΔPSE). The DSE of the U2 snRNA promoter can be thought of as a specialized enhancer, while the PSE functions much like a TATA box (9). We constructed two derivatives of the mU2 minigene, one lacking the DSE (mU2ΔDSE) and the other lacking both the DSE and the PSE (mU2ΔDSEΔPSE). Initially, *Sma*I digests of DNA from Geneticin-resistant cell lines were analyzed by genomic blotting for the presence of tandem arrays of the mU2ΔDSE and mU2ΔDSEΔPSE constructs. The mU2 probe should detect *Sma*I fragments of 1,469 (endogenous U2 genes), 753 (mU2ΔDSE), and 606 (mU2ΔDSEΔPSE) bp. To our surprise, 32 of 52 mU2ΔDSE cell lines and 35 of 65 mU2ΔDSEΔPSE cell lines had at least as many artificial U2 repeats as endogenous U2 repeats. Thus, large tandem arrays were easily obtained for the full U2 repeat (iU2) and for the transcriptionally crippled mU2ΔDSE and mU2ΔDSEΔPSE constructs, but not for the mU2 minigene (Table 1). We do not yet understand these results, but, as mentioned above, one attractive interpretation is that large mU2 arrays are unstable because the genes somehow escape dosage compensation, perhaps because they lack an additional element that is present in the full 5.8-kb repeat unit. In fact, our preliminary results indicate th[1]at we can easily obtain large artificial arrays of the mU2+CT construct (Fig. 1). The $(CT)_{70}$ dinucleotide repeat may therefore stabilize the U2 tandem array and/or participate in dosage compensation.

On the basis of the *Sma*I digestion patterns, 25 mU2ΔDSE lines and 25 mU2ΔDSEΔPSE lines were further characterized by digestion with *Bam*HI and *Bgl*II; the digests were resolved by FIGE, and the dried agarose gels were hybridized directly with the labeled probe (28). Of these, five mU2ΔDSE and six mU2ΔDSEΔPSE lines containing a single large array were

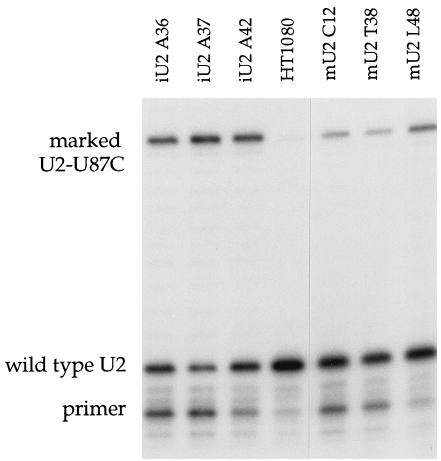


FIG. 4. Relative expression of U2 snRNAs derived from artificial and endogenous U2 tandem arrays. Total RNA from the representative cell lines indicated above the lanes was assayed by primer extension. The labeled primer was a 20-mer complementary to positions 89 to 109 of U2 snRNA (see Fig. 1B). In the presence of ddATP, the extension products derived from wild-type and marked U2-U87C snRNAs are 2 and 13 nucleotides longer than the primer, respectively.

chosen for analysis of U2 expression and fragility. Careful reanalysis of these mU2ΔDSE and mU2ΔDSEΔPSE lines by *Sma*I digestion, genomic blotting, and phosphorimager analysis (Fig. 2A) revealed that the mU2ΔDSE tandem arrays contained 22 to 112 copies, while the mU2ΔDSEΔPSE arrays contained 8 to 98 copies (Table 1).

Virally induced fragility. Cells were infected with Ad12 at a multiplicity of infection of 10 for 18 h and treated with colcemid for 3 h, and fragility was assayed both by DAPI staining and by FISH with a U2 probe. We examined at least 50 metaphases for each iU2 cell line and 100 metaphases for all other cell lines, and we always observed 80 to 90% breakage of the endogenous *RNU2* loci (Table 1). We had expected the degree of fragility to vary from one construct to another, but not the morphology of the break itself; the unexpected variety of breaks observed (Fig. 3) may be revealing of the mechanism of breakage (see Discussion).

For most cell lines containing artificial arrays of the full 5.8-kb U2 repeat (iU2), virally induced breakage of the artificial U2 locus was at least as frequent as (two lines) if not more frequent than (four lines) that of the endogenous *RNU2* loci, although the frequency of breakage was occasionally reduced to 10 to 20% (two lines). A clear gap was usually seen in both DAPI-stained sister chromatids, usually accompanied by a slight gap in the fluorescent U2 signal (Fig. 3A, lines iU2 A11 and iU2 A16). A much larger, nonstaining gap was observed in a few cases, and the fluorescent U2 signal then appeared as a series of bright beads spanning the gap (Fig. 3A, line iU2 A36). We also observed that the total U2 signal derived from both natural and artificial U2 arrays often increased upon Ad12 infection, regardless of whether the signal appeared as a single unsplit bead (Fig. 3A, line iU2 A16) or an entire necklace (Fig. 3A, line iU2 A36).

For mU2 arrays, virally induced fragility was most often expressed as a slight gap in the DAPI staining of a single sister chromatid, accompanied by splitting of the U2 signal. Occasionally, a larger gap was observed, accompanied by a dislocation in the chromosome axis; in these cases, the U2 signal was generally observed on both sides of the gap (Fig. 3B).

For mU2ΔDSEΔPSE arrays, no discernible breaks or gaps were visible in the DAPI stain, nor any splitting of the U2 signal (data not shown). The same was true for most of the mU2ΔDSE arrays, although a barely detectable gap could be seen in a small percentage of metaphase spreads with relatively extended chromosomes (Fig. 3C and Table 1). These subtle gaps are unlikely to be artifactual because no such gaps are seen in the mU2ΔDSEΔPSE arrays or in uninfected controls.

U2 snRNA expression. We assayed expression of the marked U2-U87C genes in the artificial U2 arrays of all cell lines that had been scored for Ad12-induced fragility. By using total cytoplasmic RNA as a template, endogenous U2 and marked U2-U87C snRNAs were measured simultaneously by a primer extension assay that relies on differential extension through U2 position 87 in the presence of ddATP (Fig. 1B and 4). The ratio of marked U2-U87C to wild-type U2 extension product was determined by phosphorimager analysis (Table 1, RNA ratio). Corrections were made for 2.7% readthrough by MLV reverse transcriptase on the HT-1080 wild-type U2 snRNA template (Fig. 4, lane HT1080; Table 1).

All of the iU2 and mU2 lines expressed U2-U87C snRNA efficiently, but U2 expression was nearly (mU2ΔDSE) or completely (mU2ΔDSEΔPSE) abolished by deletion of U2 transcriptional regulatory elements. We calculated the relative U2 snRNA expression per gene (Table 1, RNA/gene) by dividing the RNA ratio (U2-U87C snRNA/U2 snRNA) by the DNA ratio (U2-U87C DNA/U2 DNA). Unexpectedly, U2 expression per gene varied widely for the iU2 lines (0.31 to 1.6 relative to the endogenous U2 genes) but was nearly constant for the mU2 lines (0.9 to 1.1). These data are consistent with the possibility mentioned above that mU2, but not iU2, constructs may escape dosage compensation. We also calculated U2 expression per locus, assuming that all endogenous loci were equally expressed (Table 1). These data revealed that expression per locus does not correlate with gene copy number; rather, the site of integration of the artificial tandem array appears to be the major influence on U2 snRNA expression.

DISCUSSION

The work presented here sets the stage for a better understanding of the molecular mechanism of Ad12-induced chromosomal fragility. We find that artificial arrays of either the full 5.8-kb U2 repeat unit or an 834-bp U2 minigene are fragile but that fragility is abolished when either of two key U2 transcriptional regulatory elements—the DSE and PSE—is deleted. Unexpectedly, the degree of Ad12-induced fragility did not appear to correlate with the levels of U2 snRNA expressed by the artificial arrays. Thus, virally induced fragility requires U2 transcriptional regulatory elements, but not necessarily U2 snRNA transcription per se. We also find that the frequency of chromosome breakage is nearly independent of the number of tandem repeats in the artificial U2 arrays and that breakage of the largest artificial arrays results in a "beads-on-a-string" phenotype in which regions of condensed U2 chromatin alternate with relatively extended regions of U2 chromatin or DNA. The existence of the beads-on-a-string suggests that virally induced fragility does not spread like the heterochromatic condensation responsible for position effect variegation (see, for example, reference 38); rather, regions of fully condensed chromatin can persist although surrounded by noncondensed regions.

We were surprised that the number of U2 repeats within an artificial array had little or no effect on the degree of adenovirus-induced fragility. Of eight artificial U2 arrays containing 12 to 79 tandem copies of the full 5.8-kb U2 repeat unit (Table 1, iU2 cell lines), six (tandem arrays of 12, 29, 30, 48, 77, and 79 repeats) were at least as fragile as the natural *RNU2* locus while two (tandem arrays of 25 or 32 copies) were much less

fragile (10 to 20 versus 80 to 90% breakage of *RNU2*). Similarly, Li et al. (21) found that an artificial array containing 60 tandem copies of the full U2 repeat unit was only 60% as fragile as *RNU2*; however, the actual frequency of damage may have been underestimated in this case, because the array was integrated near or within a nucleolus organizer region for which virally induced fragility was difficult to score (21). Taken together, these results suggest that the site of integration, and not gene copy number, is the main determinant of fragility. We also suspect, as discussed below, that the fragility of artificial repeats is substantially independent of U2 gene copy number because the observed degree of fragility cannot increase once the probability of breakage approaches unity.

Knowing that three and possibly all four of the specific Ad12-induced fragile sites colocalize with clusters of abundant, ubiquitously expressed, small structural RNAs (14, 22, 23, 30, 31, 35, 47, 48, 63), we suspected that the U2 snRNA gene itself would be necessary, and perhaps sufficient, for Ad12-induced fragility. To test this idea, we generated tandem arrays of a U2 minigene (mU2) containing only those sequences known to be required for U2 snRNA expression—the 5′ transcriptional regulatory elements (2, 3, 27), the U2 snRNA coding region, and the 3′-end formation signal (1, 40). Yet after three independent ligations, transfections, and cell clonings, we obtained only three cell lines with mU2 arrays that were large enough to be detected by FISH (Table 1, mU2 cell lines). Unexpectedly, these lines contained only five, six, or eight copies of the mU2 repeat, almost an order of magnitude less than the copy numbers routinely obtained for the full 5.8-kb U2 repeat unit; moreover, the chromosomes of the lines containing five or six copies of mU2 were 5 to 10% as fragile as the resident *RNU2* locus, and that of the line with eight copies was only marginally more fragile (10 to 20%). Although these data demonstrate that the U2 minigene contains all the essential elements required to generate an Ad12-inducible fragile site, the data also imply that the full 5.8-kb U2 repeat unit contains one or more additional accessory elements that increase the efficiency of induction or the stability of the array or that render the artificial array substantially independent of chromosome position effects.

To determine whether the known U2 snRNA transcriptional regulatory elements play a role in Ad12-induced fragility, we generated derivatives of mU2 lacking one or both of the known transcriptional regulatory elements (2, 3, 27, 40, 44). The DSE, extending from positions −231 to −214 upstream of the transcription start site, binds adjacent Sp1 and Oct-1 factors and functions as a specialized enhancer sequence in collaboration with the PSE. The PSE, extending from −61 to −41, binds a TATA-binding-protein-containing complex known as SNAPc and functions somewhat like a TATA box (40). Artificial arrays of the U2 minigene lacking both transcriptional regulatory elements (mU2ΔDSEΔPSE) were not fragile, but arrays lacking only the DSE (mU2ΔDSE) occasionally exhibited small, barely detectable gaps (Fig. 3C). The frequency of these gaps did not correlate with the copy number of the mU2ΔDSE repeat unit in the artificial array. The gaps seen in mU2ΔDSE tandem arrays are unlikely to be artifactual; although Ad12 infection can induce low levels of random chromosome damage, gaps were seen in as many as 5% of the mU2ΔDSE metaphases but never in >100 mU2ΔDSEΔPSE metaphases. These data clearly indicate that U2 transcriptional regulatory elements are required for fragility, but, as discussed below, this does not necessarily mean that fragility requires U2 transcription per se. Similar conclusions have been reached by Gargano et al. (13a).

All of the artificial U2 arrays were constructed with the marked U2-U87C snRNA gene. Although U2 snRNA is highly conserved, position 87 is phylogenetically variable (61) and the U87C mutation would not be expected to have any effect on U2 snRNP assembly or function. U2-U87C snRNA could be distinguished from endogenous U2 snRNA by a simple primer extension assay (Fig. 4), and this allowed us to correlate U2 expression from the artificial arrays with virally induced fragility. For the full 5.8-kb U2 repeat (iU2 cell lines), U2-U87C snRNA expression per gene varied over a sixfold range from 30 to 200% of the wild-type level; expression per iU2 locus was always greater than wild type and sometimes approached 10-fold-higher levels. For the U2 minigene (mU2 cell lines), U2-U87C snRNA expression was slightly greater than wild type per gene but only 60 to 90% of wild type per locus. None of the promoter deletion lines expressed significant levels of U2-U87C snRNA except four mU2ΔDSE lines (containing 22, 53, 79, and 112 tandem repeats) in which expression approached 5% of wild type. Deletion of the DSE had previously been shown to result in similar levels of residual U2 snRNA expression in a transient, heterologous assay (3), but this is the first evidence that the DSE is required for efficient U2 expression in a stable, chromosomal context.

No correlation between fragility of the various artificial arrays and U2-U87C expression per gene or per locus could be discerned (Table 1). This could suggest that U2 transcriptional regulatory elements are required for fragility but not for active U2 transcription by RNA polymerase II. Alternatively, the many U2 snRNA genes within a single array might not be equally active, perhaps reflecting the morphological distinction between the beads and string (Fig. 3A; also see below). In this case, any correlation between transcription and fragility would be masked when expression levels were averaged over active and inactive genes. We are currently trying to determine whether U2 snRNA transcription regulatory factors participate directly in the molecular mechanism of Ad12-induced fragility or indirectly by stimulating transcription, delaying replication, or by affecting some other process that can interfere with chromatin condensation. Although our data are consistent with the interpretation that U2 transcription is required for fragility, it is important to remember the multifunctional nature of many key regulatory factors. For example, transcription factors have turned out to play a role in repair (10) and recombination factors have been found to play a role in transcription (15). Indeed, work on systems as diverse as meiotic recombination in *S. cerevisiae* (58) and isotype class switching in mammals (20) suggests that the ability of transcription factors to stimulate or regulate recombination need not involve transcription per se.

The morphology of the artificial fragile sites may provide some clues regarding the mechanism of virally induced fragility. In both the mU2 and the iU2 arrays, virally induced chromosome damage can range from a nonstaining gap in one sister chromatid to an apparently complete break in both sister chromatids as visualized by DAPI staining of the DNA. The U2 gene signal is sometimes split by the gap (Fig. 3A, line iU2 A24), it sometimes fills the gap (Fig. 3A, line iU2 A37), and, in some of the larger iU2 arrays, it appears as beads on a string spanning the gap in the broken chromosome arm (Fig. 3A, line iU2 A36). The invisible string represents regions of relatively noncondensed chromatin separating bright beads or clusters of more highly condensed chromatin. Assuming that the condensed chromosomes are approximately 1 μm in length overall, the invisible strings may contain as little as 300 bp (0.1 μm) of relatively uncondensed DNA while each of the bright beads would contain perhaps seven or eight U2 repeat units. Interestingly, the total U2 signal derived from the natural and ar-

tificial U2 arrays often increases upon Ad12 infection, whether the signal appears as a single unsplit bead (Fig. 3A, line iU2 A16) or an entire necklace (Fig. 3A, line iU2 A36). Thus, the U2 genes become more accessible to the biotinylated probe after Ad12 infection, and the beads (like the string) must be less condensed than in uninfected cells. The implication is that multiple U2 repeat units within each U2 array are uncondensed, and this should facilitate a detailed comparison of U2 chromatin structure in uninfected and Ad12-infected cells. We note, however, that U2 snRNA transcription might not involve large-scale chromatin decondensation, because the U2 transcription unit, unlike mRNA and rRNA transcription units which extend for many kilobases, is comparable in size to a single nucleosome and transcription termination occurs close to the 3′ end of the U2 coding region (1, 9).

Why are the mU2 arrays less fragile than tandem arrays of the full 5.8-kb iU2 repeat unit? The mU2 arrays might be too small to be fragile, but this seems unlikely since natural *RNU2* arrays containing as few as five or six copies are no less fragile than larger *RNU2* alleles (21a). The low level of mU2 fragility might reflect impaired U2 expression, but this also seems unlikely because the level of U2 expression per gene is actually higher in the mU2 artificial arrays than in the wild type, and the level of U2 expression per locus is only slightly lower than for the natural *RNU2* loci (Table 1). Strong mU2 expression also rules out promoter occlusion and/or transcriptional interference between closely spaced U2 snRNA genes. In fact, the U2 snRNA genes of *Xenopus laevis* are organized as 500 to 1,000 tandem repeats of an 831-bp unit (29), which is essentially identical in size to the mU2 repeat unit, and the sea urchin U2 genes are organized as ≈25 tandem repeats of a 1.1-kb unit (7).

Chromosome position effects could also explain the reduced level of mU2 fragility. Chromatin structure (reviewed in reference 53) can strongly modulate the expression of stably integrated exogenous genes (54) and the induction of certain fragile sites (59, 60). Thus, mU2 arrays might lack an accessory element that is present in the full 5.8-kb iU2 repeat unit and can function as an insulator (8) or scs element (53) to render artificial U2 arrays partially immune to the immediate chromosomal environment; however, the existence of two iU2 cell lines (iU2 A16 and A24) containing large arrays that are no more fragile (10 to 20%) than the mU2 cell line L48 argues against an accessory element (Table 1). Nor does the degree of fragility appear to correlate with the site of integration. Although the apparently telomeric iU2 A24 array is less fragile than most in the iU2 series, the iU2 A41 array is no further from the telomere but is normally fragile (Fig. 3A and Table 1).

Fragility could also reflect delayed DNA replication or prolonged U2 snRNA transcription. In the case of fragile X syndrome, for example, chromosome fragility is thought to reflect incomplete chromatin condensation resulting from delayed DNA replication of a pathologically expanded CGG repeat (16, 18, 34). Integration of an artificial U2 array into a late-replicating region might then enhance the intrinsic fragility of the array, while integration into an early-replicating region would tend to mask the fragile site by allowing more time for chromatin condensation. Alternatively, Ad12 infection might cause fragility by prolonging U2 snRNA transcription into S phase, when most transcription is thought to cease (references 25, 42, and 56, but see reference 64), and this might interfere with chromatin condensation either directly or indirectly by delaying DNA replication. Tandem arrays of the full 5.8-kb iU2 repeat unit might then appear to be more fragile than mU2 arrays if iU2 contained an accessory element favoring integration or maintenance of larger tandem arrays or preferential integration of such arrays into intrinsically fragile chromosome sites.

Interestingly, addition of the $(CT)_{70}$ dinucleotide repeat to mU2 (Fig. 1A, construct mU2+CT) has enabled us to readily create much larger arrays of mU2 (3a). This suggests that the $(CT)_{70}$ dinucleotide repeat may determine the site, size, and/or stability of the artificial U2 arrays. Whether the $(CT)_{70}$ dinucleotide repeat also affects transcription and/or fragility is still unknown. Normal *RNU2* alleles vary in size from 5 to 25 tandem repeats (37), and therefore U2 snRNA (like U1 [26]) must be subject to some form of dosage compensation (3a). One especially intriguing possibility is that large mU2 arrays might be unstable if mU2, but not mU2+CT, is able to escape dosage compensation.

ACKNOWLEDGMENTS

We heartily thank David C. Ward for generous use of his state-of-the-art imaging facilities, Joan Menninger and Jonathan Lieman for patient, cheerful instruction in all aspects of FISH, and Silvia Bacchetti for virus stocks and for communicating unpublished results.

This work was supported by NIH awards GM41624 and GM31073 to A.M.W.

REFERENCES

1. **Ach, R. A., and A. M. Weiner.** 1987. The highly conserved U small nuclear RNA 3′-end formation signal is quite tolerant to mutation. Mol. Cell. Biol. **7:**2070–2079.
2. **Ares, M., Jr., J.-S. Chung, L. Giglio, and A. M. Weiner.** 1987. Distinct factors with Sp1 and NF-A specificities bind to adjacent functional elements of the human U2 snRNA gene enhancer. Genes Dev. **1:**808–817.
3. **Ares, M., Jr., M. Mangin, and A. M. Weiner.** 1985. Orientation-dependent transcriptional activator upstream of a human U2 snRNA gene. Mol. Cell. Biol. **5:**1560–1570.

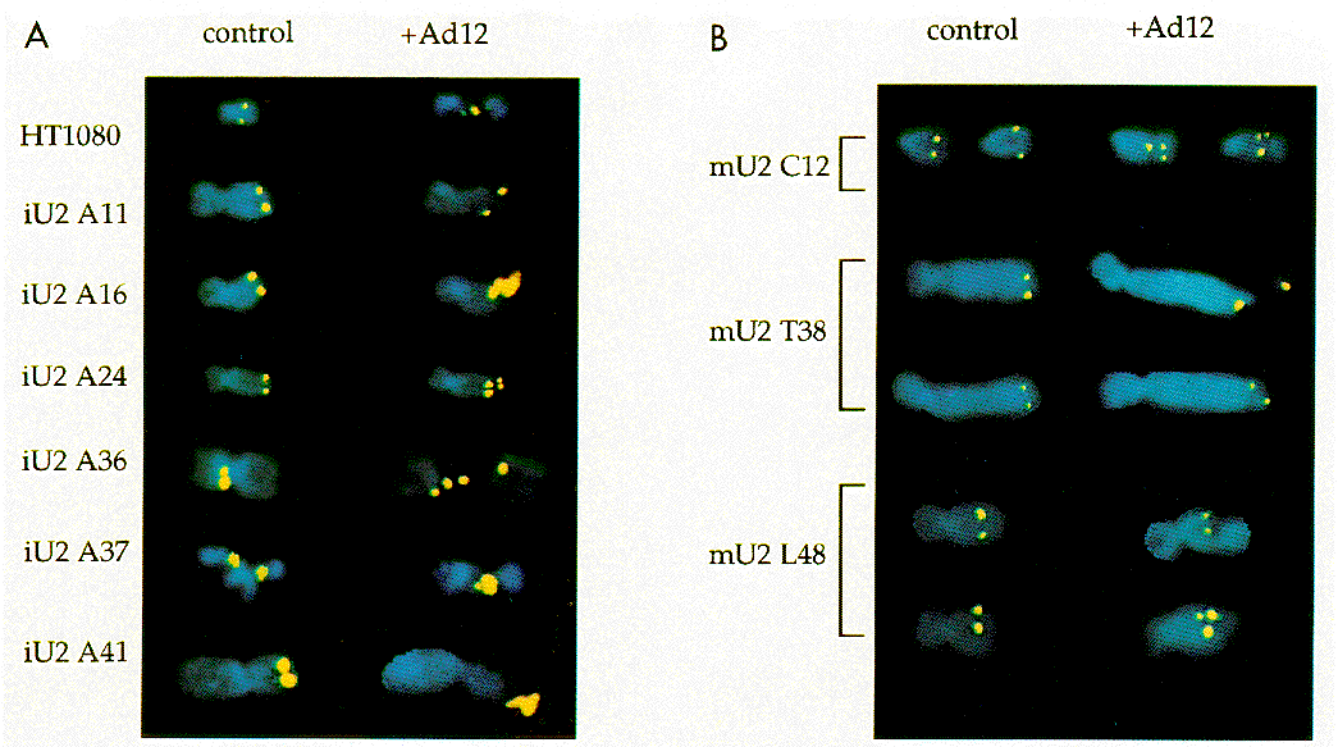
3a. **Bailey, A. D.** Unpublished data.

4. **Ballard, S. G., and D. C. Ward.** 1993. Fluorescence *in situ* hybridization using digital imaging microscopy. J. Histochem. Cytochem. **41:**1755–1759.
5. **Bloomfield, V. A.** 1991. Condensation of DNA by multivalent cations: considerations on mechanism. Biopolymers **31:**1471–1481.
6. **Caporossi, D., S. Bacchetti, and B. Nicoletti.** 1991. Synergism between aphidicolin and adenoviruses in the induction of breaks at fragile sites on human chromosomes. Cancer Genet. Cytogenet. **54:**39–53.
7. **Card, C. O., G. G. Morris, D. T. Brown, and W. F. Marzluff.** 1982. Sea urchin small nuclear RNA genes are organized in distinct tandemly repeated units. Nucleic Acids Res. **10:**7677–7688.
8. **Chung, J. H., M. Whiteley, and G. Felsenfeld.** 1993. A 5′ element of the chicken β-globin domain serves as an insulator in human erythroid cells and protects against position effect in *Drosophila*. Cell **74:**505–514.
9. **Dahlberg, J. E., and E. Lund.** 1988. The genes and transcription of the major small nuclear RNAs, p. 38–70. *In* M. Birnstiel (ed.), Structure and function of major and minor small nuclear ribonucleoprotein particles. Springer-Verlag, Heidelberg, Germany.
10. **Drapkin, R., J. T. Reardon, A. Ansari, J. C. Huang, L. Zawel, K. Ahn, A. Sancar, and D. Reinberg.** 1994. Dual role of TFIIH in DNA excision repair and in transcription by RNA polymerase II. Nature (London) **368:**769–772.
11. **Durnam, D. M., J. C. Menninger, S. H. Chandler, P. P. Smith, and J. K. McDougall.** 1988. A fragile site in the human U2 small nuclear RNA gene cluster is revealed by adenovirus type 12 infection. Mol. Cell. Biol. **8:**1863–1867.
12. **Durnam, D. M., P. P. Smith, J. C. Menninger, and J. K. McDougall.** 1986. The E1 region of human adenovirus type 12 determines the sites of virally induced chromosomal damage. Cancer cells **4:**349–354.
13. **Favre, D.** 1992. Improved phenol-based method for the isolation of DNA fragments from low melting temperature agarose gels. BioTechniques **13:**24–26.

13a. **Gargano, S., P. Wang, E. Rusanganwa, and S. Bacchetti.** 1995. The transcriptionally competent U2 gene is necessary and sufficient for adenovirus type 12 induction of the fragile site at 17q21-22. Mol. Cell. Biol. **15:**6256–6261.

14. **Hammarstrom, K., B. Santesson, G. Westin, and U. Pettersson.** 1985. The gene cluster for human U2 RNA is located on chromosome 17q21. Exp. Cell Res. **159:**473–478.
15. **Hanawalt, P. C.** 1994. Transcription-coupled repair and human disease. Science **266:**1957–1958.
16. **Hansen, R. S., T. K. Canfield, M. M. Lamb, S. M. Gartler, and C. D. Laird.** 1993. Association of fragile X syndrome with delayed replication of the

FMR1 gene. Cell **73:**1403–1409.
17. **Harnden, D. G., and H. P. Klinger (ed.).** 1985. An international system for human cytogenetic nomenclature. S. Karger, Basel.
18. **Knight, S. J. L., et al.** 1993. Trinucleotide repeat amplification and hypermethylation of a CpG island in FRAXE mental retardation. Cell **74:**127–134.
19. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
20. **Leung, H., and N. Maizels.** 1994. Regulation and targeting of recombination in extrachromosomal substrates carrying immunoglobulin switch region sequences. Mol. Cell. Biol. **14:**1450–1458.
21. **Li, Y.-P., R. Tomanin, J. R. Smiley, and S. Bacchetti.** 1993. Generation of a new adenovirus type 12-inducible fragile site by insertion of an artificial U2 locus in the human genome. Mol. Cell. Biol. **13:**6064–6070.
21a. **Li, Z.** Unpublished results.
22. **Lindgren, V., M. Ares, Jr., A. M. Weiner, and U. Francke.** 1985. Human genes for U2 small nuclear RNA map to a major adenovirus 12 modification site on chromosome 17. Nature (London) **314:**115–116.
23. **Lindgren, V., L. B. Bernstein, A. M. Weiner, and U. Francke.** 1985. Human U1 small nuclear RNA pseudogenes do not map to the site of the U1 genes in 1p36 but are clustered in 1q12-22. Mol. Cell. Biol. **5:**2172–2180.
24. **Lund, E.** 1988. Heterogeneity of human U1 snRNAs. Nucleic Acids Res. **16:**5813–5826.
25. **Lund, E., and J. E. Dahlberg.** 1992. Control of 4-8S RNA transcription at the midblastula transition in *Xenopus laevis* embryos. Genes Dev. **6:**1097–1106.
26. **Mangin, M., M. Ares, Jr., and A. M. Weiner.** 1985. U1 small nuclear RNA genes are subject to dosage compensation in mouse cells. Science **229:**272–275.
27. **Mangin, M., M. Ares, Jr., and A. M. Weiner.** 1986. Human U2 small nuclear RNA genes contain an upstream enhancer. EMBO J. **5:**987–995.
28. **Mather, M. W.** 1988. Base composition-independent hybridization in dried agarose gels: screening and recovery for cloning of genomic DNA fragments. BioTechniques **6:**444–447.
29. **Mattaj, I. W., and R. Zeller.** 1983. *Xenopus laevis* snRNA genes: tandemly repeated transcription units showing 5′ and 3′ flanking homology with other RNA polymerase II transcribed genes. EMBO J. **2:**1883–1891.
30. **McDougall, J. K.** 1970. Effects of adenoviruses on the chromosomes of normal human cells and cells trisomic for an E group chromosome. Nature (London) **225:**456–458.
31. **McDougall, J. K.** 1971. Spontaneous and adenovirus type 12 induced chromosome aberrations in Fanconi's anemia fibroblasts. Int. J. Cancer **7:**526–534.
32. **McDougall, J. K., A. R. Dunn, and K. W. Jones.** 1972. *In situ* hybridization of adenovirus RNA and DNA. Nature (London) **236:**346–348.
33. **Miro, R., I. C. Clemente, C. Fuster, and J. Egozcue.** 1987. Fragile sites, chromosome evolution and human neoplasia. Hum. Genet. **75:**345–349.
34. **Nancarrow, J. K., E. Kremer, K. Holman, H. Eyre, N. A. Doggett, D. Le Paslier, D. F. Callen, G. R. Sutherland, and R. I. Richards.** 1994. Implications of FRA16A structure for the mechanism of chromosomal fragile site genesis. Science **264:**1938–1941.
35. **Naylor, S. L., B. U. Zabel, T. Manser, R. Gesteland, and A. Y. Sakaguchi.** 1984. Localization of human U1 small nuclear RNA genes to band p36.3 of chromosome 1 by *in situ* hybridization. Somatic Cell Mol. Genet. **10:**307–313.
36. **Paranjape, S. M., R. T. Kamakaka, and J. T. Kadonaga.** 1994. Role of chromatin structure in the regulation of transcription by RNA polymerase II. Annu. Rev. Biochem. **63:**265–297.
37. **Pavelitz, T., L. Rusché, A. G. Matera, J. M. Scharf, and A. M. Weiner.** 1995. Concerted evolution of the tandem array encoding primate U2 snRNA occurs *in situ*, without changing the cytological context of the RNU2 locus. EMBO J. **14:**169–177.
38. **Pirrotta, V., and L. Rastelli.** 1994. White gene expression, repressive chromatin domains and homeotic gene regulation in *Drosophila*. Bioessays **16:** 549–556.
39. **Romani, M., A. Baldini, E. V. Volpi, I. Casciano, C. Nobile, R. Muresu, and M. Siniscalco.** 1994. Concurrent mapping of an adenovirus 5/SV40 integration site and the U1 snRNA cluster (*RNU1*) within 400 kb of the chromosome region 1p36.1. Cytogenet. Cell Genet. **67:**37–40.
40. **Sadowski, C. L., R. W. Henry, S. M. Lobo, and N. Hernandez.** 1993. Targeting TBP to a non-TATA box *cis*-regulatory element: a TBP-containing complex activates transcription from snRNA promoters through the PSE. Genes Dev. **7:**1535–1548.
41. **Schramayr, S., D. Caporossi, I. Mak, T. Jelinek, and S. Bacchetti.** 1990. Chromosomal damage induced by human adenovirus type 12 requires expression of the E1B 55-kilodalton viral protein. J. Virol. **64:**2090–2095.
42. **Segil, N., S. B. Roberts, and N. Heintz.** 1991. Mitotic phosphorylation of the Oct-1 homeodomain and regulation of Oct-1 DNA binding activity. Science **254:**1814–1816.
43. **Simonsen, C. C., and A. D. Levinson.** 1983. Isolation and expression of an altered mouse dihydrofolate reductase cDNA. Proc. Natl. Acad. Sci. USA **80:**2495–2499.
44. **Skuzeski, J. M., E. Lund, J. T. Murphy, T. H. Steinberg, R. R. Burgess, and J. E. Dahlberg.** 1984. Synthesis of human U1 RNA. II. Identification of two regions of the promoter essential for transcription initiation at position +1. J. Biol. Chem. **259:**8345–8352.
45. **Smeets, D. F. C. M., and F. A. J. M. van de Klundert.** 1990. Common fragile sites in man and three closely related primate species. Cytogenet. Cell Genet. **53:**8–14.
46. **Smith, P. P., C. L. Friedman, E. M. Bryant, and J. K. McDougall.** 1992. Viral integration and fragile sites in human papillomavirus-immortalized human keratinocyte cell lines. Genes Chromosomes Cancer **5:**150–157.
47. **Sorensen, P. D., B. Lomholt, S. Frederiksen, and N. Tommerup.** 1991. Fine mapping of human 5S rRNA genes to chromosome 1q42.11-q42.13. Cytogenet. Cell Genet. **57:**26–29.
48. **Steffensen, D. M., P. Szabo, and J. K. McDougall.** 1976. Adenovirus 12 uncoiler regions of human chromosome 1 in relation to 5S rRNA genes. Exp. Cell Res. **100:**436–439.
49. **Sutherland, G., and F. Hecht.** 1985. Oxford monographs on medical genetics, vol. 13. Fragile sites on human chromosomes. Oxford University Press, Oxford.
50. **van Arsdell, S. W., and A. M. Weiner.** 1984. Human genes for U2 small nuclear RNA are tandemly repeated. Mol. Cell. Biol. **4:**492–499.
51. **Van Arsdell, S. W., and A. M. Weiner.** 1984. Pseudogenes for human U2 do not have a fixed site of 3′ truncation. Nucleic Acids Res. **12:**1463–1471.
52. **van der Drift, P., A. Chan, N. van Roy, G. Laureys, A. Westerveld, F. Speleman, and R. Versteeg.** 1994. A multimegabase cluster of snRNA and tRNA genes on chromosome 1p36 harbours an adenovirus/SV40 hybrid virus integration site. Hum. Mol. Genet. **3:**2131–2136.
53. **Vazquez, J., and P. Schedl.** 1994. Sequences required for enhancer blocking activity of scs are located within two nuclease-hypersensitive regions. EMBO J. **13:**5984–5993.
54. **von Knebel Doeberitz, M., T. Bauknecht, D. Bartsch, and H. zur Hausen.** 1991. Influence of chromosomal integration on glucocorticoid-regulated transcription of growth-stimulating papillomavirus genes E6 and E7 in cervical carcinoma cells. Proc. Natl. Acad. Sci. USA **88:**1411–1415.
55. **Westin, G., J. Zabielski, J. Hammarstrøm, H.-J. Monstein, C. Bark, and U. Pettersson.** 1984. Clustered genes for human U2 RNA. Proc. Natl. Acad. Sci. USA **81:**3811–3815.
56. **White, R. J., T. M. Gottlieb, C. S. Downes, and S. P. Jackson.** 1995. Mitotic regulation of a TATA-binding-protein-containing complex. Mol. Cell. Biol. **15:**1983–1992.
57. **Withers-Ward, E. S., Y. Kitamura, J. P. Barnes, and J. M. Coffin.** 1994. Distribution of targets for avian retrovirus DNA integration *in vivo*. Genes Dev. **8:**1473–1487.
58. **Wu, T.-C., and M. Lichten.** 1993. Meiosis-induced double-strand break sites determined by yeast chromatin structure. Science **263:**515–518.
59. **Yunis, J. J., and W. R. Hoffman.** 1990. Nuclear enzymes, fragile sites, and cancer. J. Gerontol. **44:**37–44.
60. **Yunis, J. J., and A. L. Soreng.** 1984. Constitutive fragile sites and cancer. Science **226:**1199–1204.
61. **Zavanelli, M. I., J. S. Britton, A. H. Igel, and M. Ares, Jr.** 1994. Mutations in an essential U2 small nuclear RNA structure cause cold-sensitive U2 small nuclear ribonucleoprotein function by favoring competing alternative U2 RNA structures. Mol. Cell. Biol. **14:**1689–1697.
62. **Zhuang, Y., and A. M. Weiner.** 1989. A compensatory base change in human U2 snRNA can suppress a branch site mutation. Genes Dev. **3:**1545–1552.
63. **zur Hausen, H.** 1967. Induction of specific chromosomal aberrations by adenovirus type 12 in human embryonic kidney cells. J. Virol. **1:**1174–1185.
64. **Zwerschke, W., H. W. Rottjakob, and H. Kuntzel.** 1994. The *Saccharomyces cerevisiae* CDC6 gene is transcribed at late mitosis and encodes an ATP/GTPase controlling S phase initiation. J. Biol. Chem. **269:**23351–23356.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