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Genetics

Amplified DNA sequences in Y1 mouse adrenal tumor cells: Association with double minutes and localization to a homogeneously staining chromosomal region

(*in situ* hybridization/chromosomal anomaly/carcinogenesis)

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ABSTRACT **DNA associated with double minutes (dm) of the Y1-DM mouse adrenocortical tumor cell line has been cloned in Charon 4A and a preliminary characterization has been made of a recombinant clone, λY1dm-1, isolated from this dm DNA library [George, D. L. & Powers, V. E. (1981) *Cell* 24, 117–123]. Cloned sequences in λY1dm-1 are amplified in the genome of the Y1-DM cells. They are also amplified in the genome of a related Y1 subline (Y1-HSR), which has a homogeneously staining chromosomal region (HSR). Here we report that the amplified sequences complementary to λY1dm-1 are localized to the HSR, as determined by *in situ* hybridization. In addition, we found that a population of Y1-DM cells originally containing only dm later consisted of two cell types. Some cells retained dm; others had lost dm but gained a HSR-bearing chromosome morphologically distinct from that in the Y1-HSR cell line. Subclones isolated from this mixed culture have either dm or a HSR, but not both. Southern blotting studies revealed that genomic DNA samples from subclones with a HSR, like subclones with dm, still possess amplified copies of DNA homologous to our recombinant probe. These experiments provide direct evidence that the dm and HSRs in these Y1 cells are structurally related and further support the hypothesis that these chromosomal anomalies result from a process of gene amplification.**

We have reported previously the molecular cloning of DNA obtained from a chromosomal fraction highly enriched in small, nuclear entities termed "double minutes (dm)" in a mouse adrenal carcinoma cell line, Y1-DM (1). A recombinant clone isolated from this dm DNA library (λY1dm-1) contains insert DNA sequences that are present in 100 to 200 times greater abundance in the genome of the Y1-DM cells than in normal mouse cells or in two unrelated mouse cell lines. Moreover, these sequences are also amplified in the genome of a related Y1 cell line (Y1-HSR) which has a marker chromosome with a large "homogeneously staining region" (HSR) (1, 2). The studies reported here were initiated to determine if these amplified DNA sequences are localized to the HSR, as assayed by *in situ* hybridization to metaphase chromosomes.

Because HSRs and dm have been found almost exclusively in mammalian tumor cells and because they probably result from a process of gene amplification (1, 3–5), it is important to determine their molecular composition, their relationship to the malignant properties of the cells, and their relationship to each other. Interestingly, these two kinds of chromosomal anomaly are rarely found together in the same cell. Cytogenetic observations provide indirect evidence that HSRs and dm are related in some way. For example, a human neuroblastoma cell line has been described with two populations of cells, one containing dm and the other with a HSR-bearing marker chromosome (6). We have found that, in two related sublines of the Y1 mouse adrenocortical tumor, one subline has a large number of dm and the other has a HSR (2). The loss of dm with the simultaneous appearance of a HSR has been reported in a few cases (7, 8).

In this manuscript a similar phenomenon is described for a population of Y1 cells. Previously, the absence of specific genetic or biochemical markers for these entities in tumor cells prevented a direct assessment of the structural and functional relationships between the HSRs and dm. The availability of cloned probes specific for dm of the Y1 cells provides a means to approach this problem. The studies reported here furnish direct evidence that dm and HSRs in the Y1 cells are structurally related and further support the hypothesis that they result from a process of gene amplification.

MATERIALS AND METHODS

Cell Lines, Mouse Strains, and Cytogenetic Analysis. The growth properties, culture conditions, and detailed cytogenetic analysis of the Y1-DM and Y1-HSR cell lines have been described (2). In this report, a cell passage refers to a 1:100 dilution of cells once each week. Parental LAF_1 mice were obtained from The Jackson Laboratory. The C3H-derived A9 mouse cell line was obtained from K. Smith. The spontaneously transformed thymidine kinase-deficient (TK^-) 3T3 cells were obtained from U. Francke. Subclones derived from Y1-DM cells were selected by seeding the cells at a density of 50–100 per 100-mm Petri dish and isolating individual clones with glass cloning cylinders. Trypsin/Giemsa banding of chromosomes was carried out as described (9).

DNA Blotting and Hybridization. Restriction endonuclease-digested DNA samples were subjected to electrophoresis in 0.8–1% agarose and transferred to nitrocellulose filters (Schleicher & Schuell) according to the method of Southern (10) as modified by Jeffreys and Flavell (11). Prehybridization, hybridization, and washing of filters were as detailed (1). ^{32}P or ^{3}H labeling of DNA was carried out by nick-translation (12).

dm Isolation. The dm were isolated from 3T3 TK^- and Y1 cells by using a differential centrifugation protocol (1). Briefly, metaphase chromosome preparations were centrifuged at 4100 × *g* in an HB-4 rotor of a Sorvall RC-2B centrifuge to separate the majority of chromosomes from dm. The resulting supernatant was centrifuged at 16,000 × *g* for 60 min to pellet dm, which were resuspended in a small volume of chromosome isolation buffer.

***In Situ* Hybridization.** Slides were prepared for *in situ* hybridization by a modification of the method of Pardue and Gall

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Abbreviations: dm, double minutes; HSR, homogeneously staining region; TK, thymidine kinase; kb, kilobase(s); MTX, methotrexate.

(13). DNA was denatured by heating the slides in 15 mM NaCl/1.5 mM sodium citrate at 95–100°C for 20 sec and then dehydrated in ethanol. Hybridizations were carried out under cover slips in 50 μl of 0.45 M NaCl/0.045 M sodium citrate containing 100 mM Tris·HCl (pH 7.4), poly(rA) at 100 μg/ml, salmon sperm DNA at 1 mg/ml, and 10^4–10^5 cpm of [^{3}H]λY1dm-1. Slides were incubated at 65°C for 48 hr in a moist chamber. They were then washed extensively in 0.45 M NaCl/0.045 M sodium citrate at 60°C, dehydrated with 70% ethanol followed by 95% ethanol, and air dried. Autoradiography and Giemsa staining were carried out as described (13) with Kodak NTB-2 emulsion and Kodak D-19 developer. Exposure times were 1–3 months.

Bacteria, Bacteriophage, and Plasmids. Isolation and characterization of recombinant bacteriophage clone λY1dm-1 have been described (1). *Escherichia coli* strain DP50supF and bacteriophage were maintained and propagated according to Maniatis *et al.* (12) and Blattner *et al.* (14). The 3.8-kilobase (kb) *Eco*RI insert of λY1dm-1 was subcloned into the *Eco*RI site of alkaline phosphatase-treated (15) pBR322 according to the protocol of Lacy *et al.* (16). Recombinant plasmids were used to transform *E. coli* HB101 (17) and plasmid DNA isolated as reported (16). This work was carried out under P1-EK1 conditions as described in the National Institutes of Health guidelines for recombinant DNA research.

RESULTS

Specific Association of Amplified Sequences with Y1 dm. Recombinant clone λY1dm-1 contains insert *Eco*RI fragments of 3.8, 3.0, and 2.9 kb which are amplified in the genome of the Y1 cells. As determined by Southern blotting studies, sequences hybridizing to λY1dm-1 are present, but are not amplified, in the genome of two unrelated mouse cell lines, A9 and 3T3 TK$^-$ (1). The cloned DNA in λY1dm-1 was derived from a chromosomal fraction highly enriched in dm of the Y1-DM cell line. This chromosomal fraction was obtained by a protocol involving differential centrifugation of metaphase chromosomes, as described (1). We wished to obtain additional evidence that the cloned inserts are indeed specifically associated with the Y1 dm and are not derived from other genetic material possibly contaminating the dm preparations. Therefore, we looked for the presence of λY1dm-1 related sequences in chromosomal fractions derived in the same way from the A9 and 3T3 TK$^-$ cell lines. The A9 cells do not contain dm or HSRs; the 3T3 TK$^-$ cells contain an average of 130 dm per cell.

For the studies described here, we used as our probe the plasmid pYd1-1 which contains the 3.8-kb insert of λY1dm-1. The 3.8-kb fragment was chosen for these studies because we have evidence that it contains sequences complementary to specific RNA transcripts in the Y1 cells (unpublished data). DNA was prepared from dm preparations of the Y1-DM and 3T3 TK$^-$ cell lines and from a chromosomal fraction isolated in the same way from the A9 cell line. Because the A9 cells do not have dm, this fraction contains a small amount of chromosomal material and mitochondrial DNA presumably similar to that contaminating the dm preparations. The DNAs were digested with *Eco*RI and analyzed by filter hybridization for the presence of sequences homologous to ^{32}P-labeled pYd1-1. Bands of hybridization were detected only in that chromosomal fraction enriched for the dm of the Y1-DM cells (Fig. 1). No bands were detected with equivalent amounts of DNA from the A9 and 3T3 fractions. Similar results were obtained when λY1dm-1 was used as a probe (data not shown). We conclude that, in the Y1-DM cells, amplified copies of DNA homologous to pYd1-1 (and λY1dm-1) cosediment with, and are presumably derived from, dm.

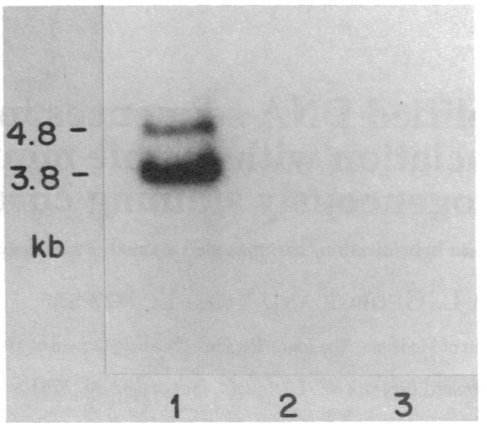

FIG. 1. Association of pYd1-1 sequences with Y1 dm. ^{32}P-Labeled pYd1-1 was hybridized to a Southern blot of *Eco*RI-digested DNA (0.1 μg) from a chromosomal fraction enriched in dm of Y1-DM cells (lane 1), 3T3 TK$^-$ cells (lane 3), and a comparable chromosomal fraction from A9 cells (lane 2).

Localization of Amplified Sequences to the HSR. Fig. 2*A* is a representative trypsin/Giemsa-banded metaphase spread of the Y1-HSR cell line, showing the marker chromosome with the HSR. Such chromosomal regions, which fail to exhibit the differential staining pattern characteristic of trypsin/Giemsa banding but stain more uniformly, have been referred to as HSRs (18). The distinctive appearance of this marker chromosome in the Y1-HSR cells, including its size, presence of a short arm, and centromeric position (2), makes it easily identified even in unbanded preparations.

Because insert sequences of λY1dm-1 were amplified in genomic DNA from Y1-HSR cells, we wished to establish whether such sequences are present within the HSR. To do this, ^{3}H-labeled λY1dm-1 was used as a probe for *in situ* hybridization to metaphase chromosomes. The silver grains were specifically clustered along the length of the HSR (Fig. 2 *B* and *C*). There was only a random distribution of grains over all other chromosomes. Also, there was only a random labeling of the short arm and the most distal portion of the long arm of the HSR-bearing marker chromosome. These two regions are not "homogeneously staining." In one metaphase spread, which had two morphologically indistinguishable copies of this HSR-bearing marker chromosome, both HSRs were heavily labeled (data not shown). Detailed examination of 13 metaphase spreads revealed an average of 10.9 grains over the HSR compared to an average of 0.61 grain over each of the other chromosomes. Therefore, amplified DNA sequences hybridizing to the λY1dm-1 probe are localized to the HSR in these cells.

Simultaneous Loss of dm and Appearance of a HSR. Routine chromosome analysis of our original Y1-DM cell line over a period of 2 years in continuous culture had confirmed the presence of dm in all metaphase spreads examined. However, during the course of these studies we noted a change in the chromosome complement of a subline of Y1-DM cells, previously isolated for other reasons. A second cell population appeared which no longer had dm but did have a HSR. Representative trypsin/Giemsa-banded metaphase spreads of the HSR- and dm-containing cells in this population are shown in Fig. 3. The newly appearing HSR is associated with a mouse chromosome 2 (Fig. 3*A*), and this marker chromosome has been designated H2. Except for the presence of either dm or a HSR, the chromosome complement is the same in the two cell populations. The marker chromosome H2 is morphologically distinct from the HSR-marker chromosome of the Y1-HSR cells (Fig. 2A; ref.

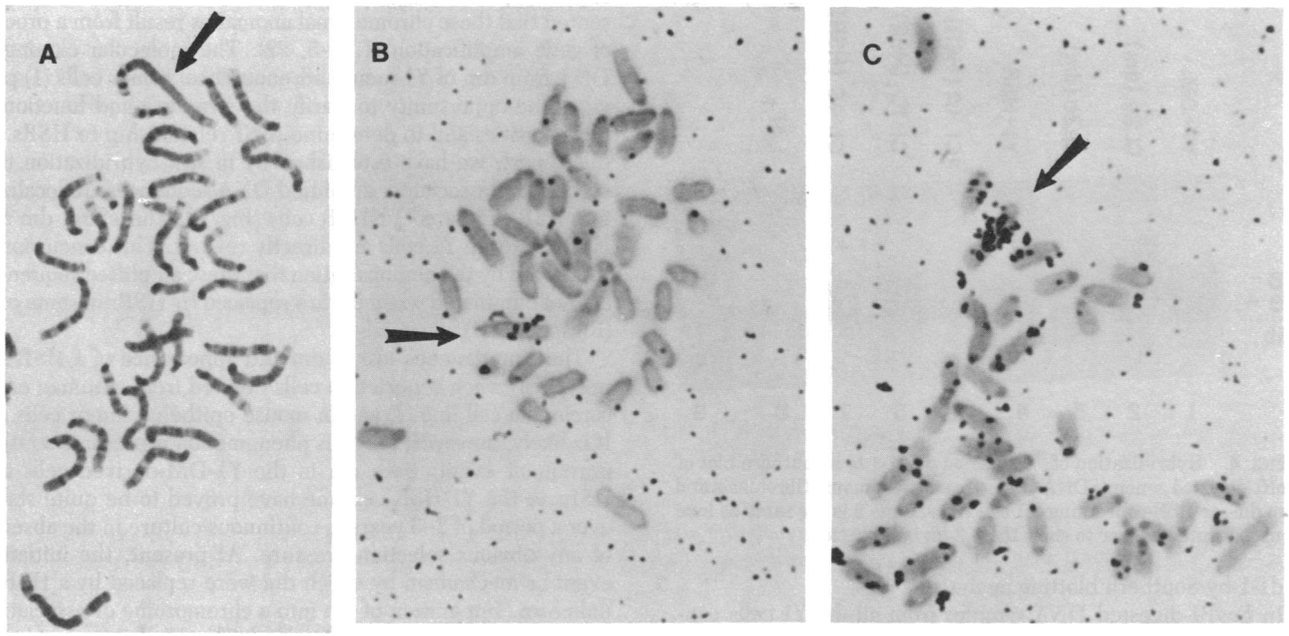

FIG. 2. Metaphase chromosome spreads of Y1-HSR cell line. Arrows indicate marker chromosome with HSR. (*A*) Trypsin/Giemsa-banded chromosomes. (*B* and *C*) *In situ* hybridization of ^{3}H-labeled λY1dm-1 probe. (Exposure times, 1–3 months.)

2). Moreover, all of these Y1-DM-derived cells could be distinguished from the Y1-HSR cells by virtue of other marker chromosomes specific for each of these cell lines, thus ruling out contamination as a source of the observed heterogeneity. A detailed cytogenetic analysis of the Y1-DM and Y1-HSR cell lines has been reported (2).

When first discovered, the HSR chromosome (H2) was present in 27 of 100 metaphases examined. The remaining cells contained dm. When assayed eight passages later, 54 of 100 cells contained a HSR. After an additional 14 passages, 90 of 100 metaphases contained the HSR, 9 had the HSR plus a small number of dm (less than six per cell), and only 1 metaphase spread contained dm in the absence of a HSR.

Abundance of DNA Sequences Homologous to pYd1-1 in dm- and HSR-Containing Subclones. The availability of the pYd1-1 recombinant plasmid allowed us to determine if sequences complementary to the insert were still present and amplified in those cells that had lost dm and gained the HSR. If the HSRs and dm in the mixed cell population were not related but were quite distinct in molecular composition, then loss of dm should result in the coincident loss of amplified pYd1-1 related DNA sequences. To distinguish between these possibilities, we isolated from the mixed cell population subclones that had dm without a HSR, as well as subclones with only the HSR. Genomic DNA was isolated from three HSR-containing subclones (Cl.1A; Cl.1C; Cl.4C) and from two dm-containing subclones (Cl.2a; Cl.3b). The DNA samples were compared to samples from the original Y1-DM cell line containing only dm, from Y1-HSR cells, and from parental LAF_1 mouse cells for the presence and relative abundance of sequences hybridizing with

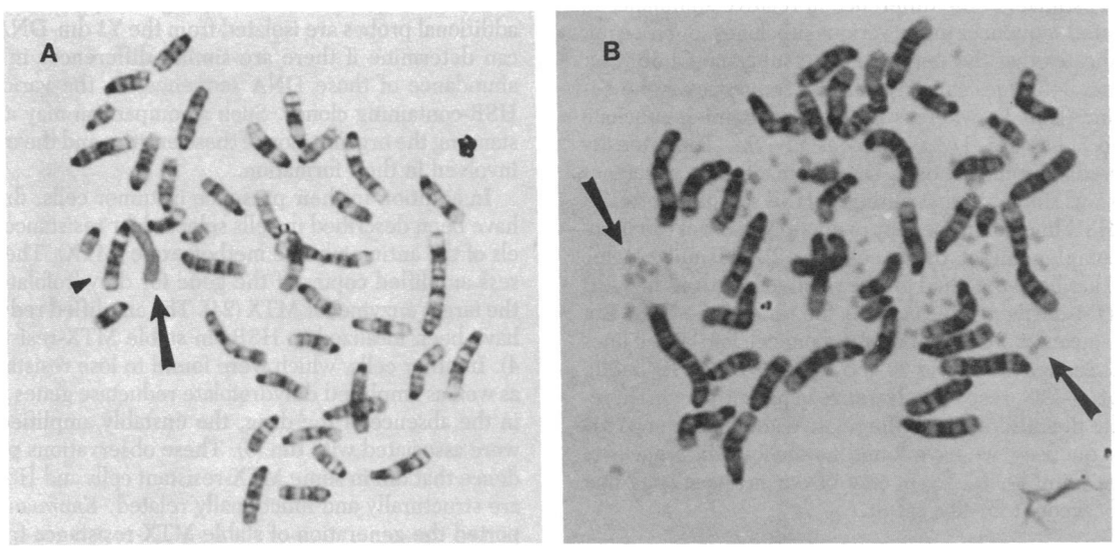

FIG. 3. Representative trypsin/Giemsa-banded metaphase spreads from dm- or HSR-containing cells derived from Y1-DM subline. (*A*) Large arrow points to HSR, which is attached at the centromere to a mouse chromosome 2 (arrowhead). (*B*) Arrows indicate representative dm.

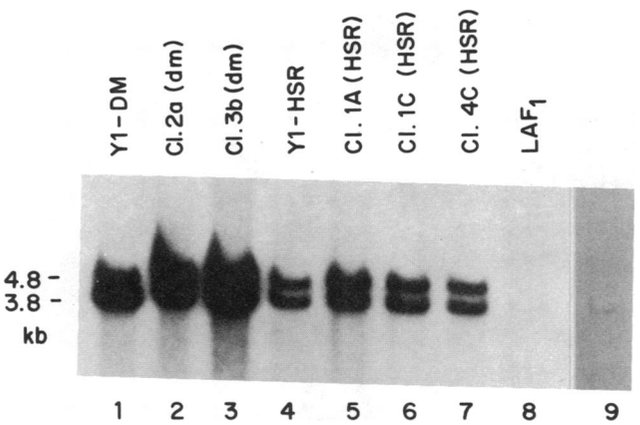

FIG. 4. Hybridization of ^{32}P-labeled pYd1-1 to a Southern blot of *Eco*RI-digested genomic DNA (10 μg) from LAF_1 mouse fibroblasts and from dm- or HSR-containing Y1 cell lines. Lane 9 is the same as lane 8 but is printed darker to show the 3.8-kb band better.

pYd1-1 by Southern blotting analysis.

In *Eco*RI-digested DNA samples from all the Y1 cells containing either dm or HSRs, the pYd1-1 probe hybridized to 4.8- and 3.8-kb fragments (Fig. 4). On a shorter exposure of the autoradiogram, the hybridization signal in lanes 1–3 clearly resolved into two bands, as seen in lanes 4–7. These fragments were the same size as those in *Eco*RI-digested DNA made from the Y1 dm-enriched chromosome fraction (Fig. 1). As in our earlier studies (1), hybridization to the 3.8-kb fragment in DNA from normal (LAF_1) mouse cells (Fig. 4, lanes 8 and 9) produced a significantly weaker signal than that seen with DNA from all of the Y1 samples. These results show that the subclones that have a HSR, like those with dm, contain amplified copies of DNA related to the 3.8-kb insert of pYd1-1. As before, the 4.8-kb band was not detected under these conditions in LAF_1 DNA (1).

The intensity of the hybridization signal was reproducibly stronger in DNA from subclones with dm compared to subclones with the HSR (Fig. 4), indicating that there are some differences in the relative abundance of the amplified sequences in the different cell lines. From the signals obtained when different amounts of *Eco*RI-digested genomic DNA from the Y1-DM cells were assayed by filter hybridization (1), we can get a rough estimate of the difference in relative abundance of pYd1-1 related sequences in the various subclones. Such a comparison indicates that the dm-containing subclone Cl.3b (Fig. 4, lane 3) has 3–5 times as many of these sequences as the Y1-DM cell line (lane 1), whereas the HSR-containing subclone Cl.4C (lane 7) has 1/5–1/3 as many as Y1-DM. The intensity of the autoradiographic bands in DNA from all three subclones with HSRs was similar to that in the Y1-HSR sample. The HSR in the Y1-HSR line comprises approximately 4.2% of the chromosome complement of these cells (1, 2). A similar value (4.2–4.7%) has been obtained for the relative length of the HSR in the Y1-DM-derived subclones. At the time of the DNA isolations, the mean (± SD) number of dm per cell for the cell lines Y1-DM, Cl.2a, and Cl.3b was 69 ± 57 (6–220), 32 ± 19 (3–80), and 27 ± 24 (4–130), respectively (range in parentheses). Therefore, despite the differences in the relative abundance of pYd1-1 related sequences, we have found no obvious differences in the average number, range, or size of dm in these cells that could easily account for this result.

DISCUSSION

HSRs and dm have been described in various tumor cell types of human and animal origin (19–22). Evidence has been presented that these chromosomal anomalies result from a process of gene amplification (1, 3–5, 22). The molecular cloning of DNA from dm of Y1 mouse adrenocortical tumor cells (1) provides the opportunity to clarify the structure and function of these entities and to determine their relationship to HSRs. In this report, we have established by *in situ* hybridization that specific dm-associated, amplified DNA sequences are localized to a HSR in related Y1-HSR cells (Fig. 2). Therefore, dm and HSRs in these Y1 cells are directly related. This conclusion is supported by the demonstration that these amplified sequences are not eliminated when dm are replaced by HSRs in some cells (Figs. 3 and 4).

The simultaneous loss of dm and appearance of a HSR has previously been reported in cells derived from a human colon carcinoma cell line (7) and in mouse epithelial tumor cells (8). It is likely, however, that this phenomenon represents a rather infrequent event. Both dm in the Y1-DM-derived cells and HSRs in the Y1-HSR cell line have proved to be quite stable over a period of 2–3 years in continuous culture in the absence of any obvious selection pressure. At present, the initiating event or mechanism by which dm were replaced by a HSR is unknown. Integration of dm into a chromosome or association with a centromere could be involved. The dm show no evidence of centromeric structure and are distributed randomly to daughter cells at mitosis (23); they often vary in number from cell to cell. If amplification of certain DNA sequences associated with dm provides cells with a selective growth advantage, then loss of dm would eliminate this advantage. On the other hand, integration of amplified sequences into a centromere-containing chromosome would ensure their equal distribution at mitosis. The rapidity with which HSR-containing cells replaced those with dm in the mixed culture of Y1-DM cells indicates that the HSR provides cells with an advantage over their dm-containing counterparts, at least *in vitro*. This observation is consistent with previous results obtained in cell mixing experiments: when equal numbers of Y1-HSR and Y1-DM cells are mixed in the same flask, Y1-HSR cells quickly predominate, despite the fact that the cell lines have similar doubling times (2).

We cannot yet account for the variation in the relative abundance of pYd1-1 related sequences in Y1-DM and our two dm-containing subclones (Fig. 4). It is possible that the dm are somewhat heterogeneous in molecular composition and that in subclones 2a and 3b we have enriched for dm having a greater number of copies of the 3.8-kb fragment present in pYd1-1. As additional probes are isolated from the Y1 dm-DNA library, we can determine if there are similar differences in the relative abundance of those DNA sequences in the various dm- and HSR-containing clones. Such a comparison may aid in understanding the organization of these entities and the mechanism(s) involved in their formation.

In addition to their presence in tumor cells, dm and HSRs have been described in cells selected for resistance to high levels of the antimetabolite methotrexate (MTX). These cells possess amplified copies of the gene for dihydrofolate reductase, the target enzyme for MTX (24). The amplified reductase genes have been localized to HSRs in stable MTX-resistant cells (3, 4). In other cells, which were found to lose resistance to MTX as well as amplified dihydrofolate reductase genes when grown in the absence of the drug, the unstably amplified sequences were associated with dm (5). These observations provided evidence that dm in some MTX-resistant cells and HSRs in others are structurally and functionally related. Kaufman *et al.* (5) reported the generation of stable MTX-resistance from unstably resistant cells, occurring with the loss of dm and a stepwise fixation of dihydrofolate reductase sequences. It would be interesting to determine whether this involved the appearance of a

HSR. In contrast to HSRs and dm specifically induced during the development of drug resistance, such as in the MTX-resistant cell lines, HSRs and dm described in most other mammalian tumor cells are present stably under no obvious selection pressure. Therefore, it is important to determine if amplified sequences associated with these entities play a role in producing or maintaining malignant transformation, or whether they may be related to the expression of specific differentiated properties in these cells. Our studies indicate that dm (or HSRs) from different transformed cells need not be identical in sequence. DNA sequences amplified in the genome of the Y1 cells are not amplified in dm-containing 3T3 TK^- cells (1). Conversely, dm-associated sequences amplified in the genome of the 3T3 cells are not amplified in the Y1 cells (unpublished data). If amplification of normal cellular genes is associated with malignant transformation, the particular gene involved may be specific to a certain cell type or pathway of differentiation (19, 25). The availability of a cloned library of dm DNA from both Y1 and 3T3 cells should make possible a direct test of this hypothesis.

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