Volume 16 Number 1 1988 Nucleic Acids Research

Hitch-hiking from HRAS1 to the WAGR locus with CMGT markers

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Received October 16, 1987; Revised and Accepted December 1, 1987

ABSTRACT

The clinical association of Wilms' tumour with aniridia, genitourinary abnormalities and mental retardation (WAGR syndrome) is characterised cytogenetically by variable length, constitutional deletion of the short arm of chromosome 11, which always includes at least part of band 11p13. HRAS1-selected chromosome mediated gene transfer (CMGT) generated a transformant, E65-6, in which the only human genes retained map either to band 11p13 or, with HRAS1, in the region 11p15.4-pter. Human recombinants isolated from E65-6 were mapped to a panel of five WAGR deletion hybrids and two clinically related translocations. We show that E65-6 is enriched ≈400-fold for 11p15.4-pter markers and ≈200-fold for 11p13 markers. 'Hitch-hiking' from HRAS1 with CMGT markers has allowed us to define seven discrete intervals which subtend band 11p13. Both associated translocations co-locate within the smallest region of overlap for the WAGR locus, which has been redefined by identifying a new interval closer than FSHB.

INTRODUCTION

In an earlier study (1), we described the establishment and cytogenetics of lymphoblastoid cell lines derived from several WAGR patients, the segregation of deleted from normal chromosomes 11 in somatic cell hybrids and their characterisation with defined gene markers. We showed that CAT and the cell surface markers MIC4 and MIC11 all map to band 11p13 and are frequently but not ubiquitously deleted in WAGR patients.

In a parallel study (2), we described the development of the chromosome mediated gene transfer (CMGT) technique with selection for expression of the activated HRAS1 oncogene, mapping at 11p15.4-pter. This procedure facilitates the stable isolation of human chromosome 11 fragments in mouse C127 cells. However, intrachromosomal rearrangement accompanies the CMGT process such that markers centromere proximal to HRAS1 can be co-transferred while intervening markers are lost.

Most recently (3), we described the isolation and sub-chromosomal localisation of forty-four human DNA recombinants from one HRAS1-CMG transformant, E67-1. This transformant contains ≈50Mbp of human DNA inserted interstitially

at a single site on a mouse chromosome (4). Several chromosome 11 markers had been co-transferred, including ones known to flank either side of the WAGR locus (2). Mapping of the E67-1 derived recombinants to our panel of WAGR deletions (now extended to five patients, three of which retain part of band 11p13), together with two clinically associated 11p13 translocations, allowed us to define ten discrete intervals on the short arm of chromosome 11 (3). These included a centromere distal interval previously defined only by FSHB (5,6) and a centromere proximal interval previously defined by CAT and MIC11 (1,6). Thus, the CMGT approach rapidly generated a valuable and extensive set of anonymous DNA markers for fine-structure mapping of the short arm of chromosome 11. The enrichment for 11p13 markers was substantial and significantly higher than would be expected from a chromosome 11 only hybrid or from a flow sorted, chromosome 11 enriched library. However, although three new intervals on the short arm of chromosome 11 were defined, none were closer to the WAGR locus than the FSHB or the CAT/MIC11 intervals. The success of the clone isolation and mapping exercise with E67-1 encouraged us to test other HRAS1-selected CMGT's, in particular those for which we had evidence for complex but potentially adventitious intrachromosomal rearrangement.

The HRAS1-selected CMG transformant E65-6 contains ≈10Mbp of human DNA (2). L1 "fingerprinting" provides evidence for complex but stable amplification and rearrangement of the isolated human DNA sequences (2). *In situ* hybridisation analysis demonstrates the presence of three discrete blocks of human chromatin, each carrying a copy of the HRAS1 oncogene (4,7). Of particular relevance to the present study, only LDHA, FSHB and MIC11, of all the chromosome 11 genes tested, have been co-transferred along with HRAS1 (Table 1). Furthermore, several of the E67-1 derived recombinants which map to band 11p13 cross-hybridise to E65-6 (Porteous et al., in prep.). E65-6 should therefore provide an ideal further test of the CMGT approach, not only for isolating chromosomal segments which directly flank the selected locus (8,9), but also for cloning syntenic sequences originally at a distance (CMGT "hitch-hiking").

MATERIALS AND METHODS

Chromosome 11 somatic cell hybrids

The human-mouse cell hybrids 1W1.LA4.9 and 1B.8.1.6 retain chromosome 11 as their sole human component (1,6). We have tested over 150 gene specific and anonymous DNA markers on chromosome 11 and all are present in 1W1LA4.9, whereas 1B.8.1.6 appears to have a terminal deletion of short arm material,

Table 1. CHROMOSOME 11 GENES IN E65-6 AND WAGR RELATED HYBRIDS.

CELL LINE	HRAS1	INS	HBB	PTH	CALC1	LDHA	FSHB	MIC11	CAT	MIC4	PGA	CLG	MIC8	APOA1
EJ-18-8D	+	+	+	+	+	+	+	+	+	+	+	+	+	+
IWI.LA4.9	+	+	+	+	+	+	+	+	+	+	+	+	+	+
IB.8.1.6.	-	+	+	+	+	+	+	+	+	+	+	+	+	+
E65-6	+	-	-	-	-	+	+	+	-	-	-	-	-	-
NYX3.1	+	+	+	+	+	+	-	-	-	-	+	+	+	+
GOX2	+	+	+	+	+	+	-	-	-	-	+	+	+	+
SAX3.10	+	+	+	+	+	+	+	-	-	-	+	+	+	+
MAX15	+	+	+	+	+	+	+	-	-	+	+	+	+	+
ANX3.10	+	+	+	+	+	+	-	+	+	+	+	+	+	+
SMX13	+	+	+	+	+	+	+	-	-	-	-	-	-	-
POR11	+	+	+	+	+	+	+	-	-	-	-	-	-	-
POR4	-	-	-	-	-	-	-	+	+	+	+	+	+	+

HRAS1, c-Harvey-ras-1; INS, insulin; HBB, β-globin; PTH, parathyroid hormone; CALC1, calcitonin; LDHA, lactate dehydrogenase A; FSHB, follicle stimulating hormone, β-subunit; CAT, catalase; PGA, pepsinogen A; CLG, collagenase; APOA1, apolipoprotein A1; MIC11, MIC4 and MIC8, cell surface markers recognised respectively by monoclonals 163.A5, F10.44.2 and 4F10.

being negative for HRAS1 (Table 1) and tightly linked anonymous DNA markers. The WAGR deletion hybrids NYX3.1, GOX2, ANX6.14, SAX3.10 and MAX15 have been described in detail elsewhere (1,3,6). The deletions in NYX3.1 and GOX2 are indistinguishable at the level of defined gene marker analysis (Table 1), but cytogenetic analysis shows that the centromere distal break in GOX2 occurs in band 11p14 whereas in NYX3.1 it extends to band 11p15.4 (1). Molecular analysis with anonymous HRAS1-CMGT derived human recombinants confirms and refines the cytogenetic interpretation (3). The deletions in ANX6.14, SAX10 and MAX15 are smaller and each retain part of band 11p13 (3,6). The reciprocal 2p11:11p13 translocation hybrids POR4 and POR11 were established from a neonate with Potter facies and genitourinary dysplasia, with bilateral undescended testes and urethral and uretral atresia, as described previously (3,6). The SMX13 hybrid carries the 11p13-11pter portion of a reciprocal translocation which segregates with aniridia in a Finnish family (10) and has been described elsewhere (3,6). The LHV hybrids (5) are derived from the same patient. E65-6 is one of a series of HRAS1-selected chromosome mediated gene (CMG) transformants, derived by transfection of mouse C127 cells with mitotic chromosomes isolated from the human EJ bladder carcinoma cell line, as described previously (2). Table 1 summarises the mapping data with respect to defined genes for each of the hybrid cell lines.

Genomic library construction, probe isolation and mapping

Genomic library construction and screening, probe isolation and mapping were essentially as described previously (3,11). Sau3A partial digest genomic

libraries of E65-6 were constructed in the lambda cloning vector EMBL3 and the cosmid cloning vector pJB8, using standard protocols. The libraries were screened at moderate density with total human DNA as radiolabelled probe and positive colonies or plaques counter-screened with radiolabelled total mouse DNA. Cosmid DNA was prepared from liquid cultures using standard protocols. Lambda DNA was prepared from cleared plate lysates using LambdaSorb (Promega Biotec) which gave consistently good yields of nick-translatable DNA. Cosmid and lambda human DNA recombinants were radiolabelled by nick translation to a specific activity of $\simeq 2 \times 10^8$cpm per μg. The repeated DNA element was removed from the hybridisation reaction by pre-annealing with 100μg unlabelled total human DNA (3,11). The recombinants were mapped by hybridisation to panels of WAGR deletion and translocation DNA's which had been digested with BamHI (5μg per track), electrophoresed through 0.8% agarose and transferred to Hybond N (Amersham Plc) membranes, according to the manufacturers instructions. Hybridisation was in 5xSSC, 5xDenhardt's, 0.5% SDS with 10% dextran sulphate or PEG 1500, at 68°C. Filters were washed to 0.1% x SSC in 0.1% SDS, 0.1% sodium pyrophosphate at 68°C and exposed to autoradiographic film at -70°C for 1-7 days.

RESULTS

19 cosmid and 35 lambda human DNA recombinants from E65-6 were localised to chromosome 11, as illustrated in Figure 1. A majority of the E65-6 derived human DNA recombinants detect sequences which show varying degrees of amplification and/or rearrangement in the E65-6 transformant compared with the chromosome donor cell line, EJ-18-8D (Fig. 1a). This is consistent with the prediction of rearrangement based on L1 'fingerprinting' (2) and in situ hybridisation analysis (4,7). It contrasts with only one genomic rearrangement out of forty-four recombinants derived from the E67-1 transformant (3), where the molecular and physical characterisation had suggested minimal molecular scrambling (2,4,7).

Recombinants found to be deleted from NYX3.1, which encompasses all of our remaining deletions (3), were further sub-localised by mapping to the smaller WAGR deletion hybrids and the associated translocations, as illustrated in Figure 2 and summarised in Figure 3. None of the E65-6 derived recombinants map below the centromere proximal breakpoint in NYX3.1. CosE65-6-3 (see Fig. 1b) and λE65-6-103 both map to the most distal deletion region. λE65-6-10A (corresponding to the higher mobility hybridising fragment in Fig. 2b) maps one interval closer to the WAGR locus, in an interval

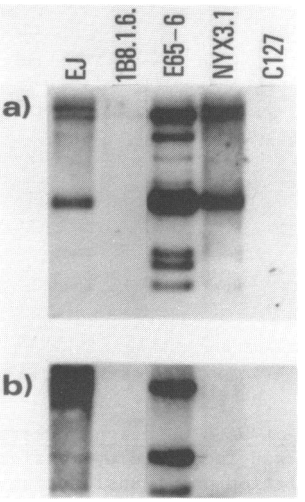

Figure 1. Identification of WAGR deletion markers. E65-6 derived human recombinants were hybridised to panels of chromosome 11 hybrid DNA's which included the WAGR deletion hybrid NYX3.1. a) λE65-6-16; b) cosB65-6-3. See the Materials and Methods section for details.

previously only defined by the E67-1 derived recombinant λP5B2.B (3).

All 11 remaining E65-6 derived WAGR deletion clones map to one or other of 5 intervals which subtend band 11p13. CosE65-6-13, cosE65-6-22B and λE65-6-128 map in the most centromere proximal deletion region, previously defined only by the E67-1 derived recombinant cosE67-1-1 (3). CosE65-6-22A and λE65-6-97 map to the same interval as the cell surface marker MIC4 (6) and the E67-1 derived recombinant λP11F9 (3). CosE65-6-6, λE65-6-84 and λE65-6-96 (see Fig. 2a) map immediately centromere proximal to the WAGR locus, with the E67-1 markers λP2G4 and λP4F11 (3), in the MIC11/CAT interval (6). CosE65-6-8 maps to the centromere distal interval previously defined by FSHB (5,6) and by the E67-1 marker λP12C9 (3). Finally and most importantly, both λE65-6-74 and λE65-6-10B (corresponding to the lower mobility hybridising fragment in Fig. 2b) map to a new interval, immediately centromere distal to the SMX13 and the POR11 translocation breakpoints, but centromere proximal to λP12C9 and FSHB. These results refine and redefine the extent of the smallest region of overlap for the WAGR locus.

It is of interest that λE65-6-10 hybridises to two discontinuous regions on the short arm of chromosome 11 (Fig. 2b and Fig. 3). Further analysis will be necessary to determine whether this is accountable by complex rearrangement

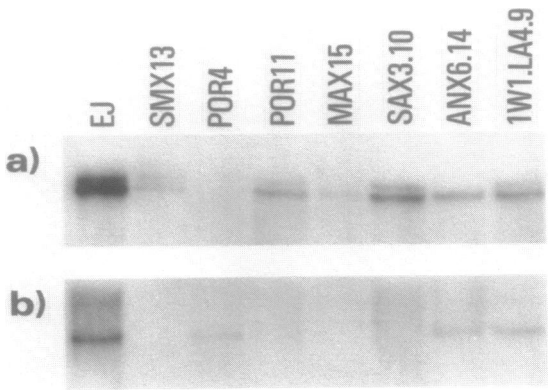

Figure 2. Sub-localisation of WAGR deletion markers. Recombinants mapping to the NYX3.1 deletion were sub-localised by hybridisation to an extended panel of WAGR associated deletion and translocation hybrids, as described in the Materials and Methods section. a) λE65-6-96; b) λE65-6-10.

within E65-6, bringing normally disparate DNA segments together, or whether it truely reflects the existence of duplicated DNA segments on the short arm of chromosome 11. The E67-1 derived recombinant λP5B2 positively identifies two distinct DNA segments which span the centromere distal breakpoint in GOK2 (3). A second E65-6 derived recombinant, cosE65-6-22, also hybridises to two distinct regions of the chromosome 11 short arm (Figure 3).

DISCUSSION

The observation of chromosomal rearrangement accompanying the chromosome transfer process prompts us to question the validity of this approach as a first order mapping tool (12,13). However, the power of CMGT as an enrichment cloning strategy for markers immediately flanking the locus under selection has been clearly demonstrated (8,9,13). Our results strongly suggest that even those transgenomes which have undergone extensive and complex rearrangement still consist largely, if not exclusively, of sequences syntenic with the locus under selection. There is also a strong tendency for markers tightly linked on the donor chromosome to co-segregate. All forty-four of the recombinants derived from the HRAS1-CMG transformant E67-1 map to chromosome 11 (3). All fifty-four of the E65-6 derived recombinants described here, not only map to chromosome 11, but localise to predicted sub-regions on the short arm.

Chromosome 11 is estimated to comprise ≃4.8% of the haploid genome, equivalent to ≃140Mbp (14). It follows that E65-6 is enriched ≃200-fold

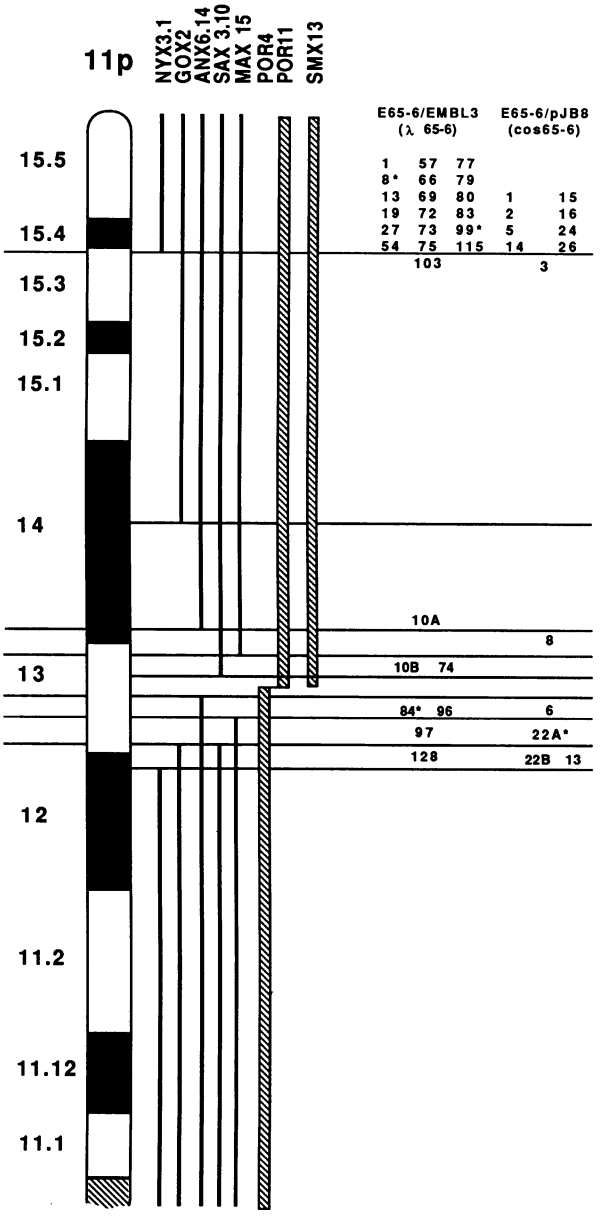

Figure 3. A physical map of the short arm of chromosome 11 and the WAGR region. The figure summarises the mapping of 19 cosmid and 35 lambda human recombinants isolated from the HRAS1-selected CMGT E65-6 to 5 independent WAGR deletions and 2 WAGR associated translocations, as described in the text. Asterisked clones show significant cross-hybridisation to mouse DNA at high stringency and may therefore identify coding sequences.

for band 11p13 markers and ≈400-fold for 11p15.4-pter markers, on the reasonable assumption that each region comprises ≈3.5% of the whole chromosome, or ≈5Mbp. The CMGT approach therefore compares favourably with other enrichment cloning strategies, such as flow sorted chromosome libraries (15), microdissection of mitotic chromosome spreads (16) or subtractive hybridisation protocols for deletion cloning (17). Success with the subtractive hybridisation strategy, moreover, has so far been restricted to the favourable condition of hemizygous deletion of the X chromosome in males (17) and may not be feesible for autosomal conditions. The idea of using CMGT associated intrachromosomal rearrangment to "hitch-hike" from the locus under selection to distant but syntenic loci introduces an important new aspect to this cloning.

Indeed, as serendipitous intrachromosomal deletion appears to be a general CMGT-associated phenomenon (2,3,13,18), the quest for a selectable marker tightly linked to the chromosomal region of interest, while still the ideal, should not limit the CMGT approach to enrichment cloning. With careful assessment of syntenic marker co-transfer patterns, it may be possible to cover each chromosome quite effectively using only a few selectable markers as "hitch-hike" start points.

The discontinuous co-transfer of chromosome 11 markers in E67-1 in the absence of significant molecular scrambling suggests a process of simple intrachromosomal deletion and religation (3). The situation in E65-6 is more complex. Hybridisation analysis with E65-6 derived human recombinants provides clear evidence for extensive rearrangement and local amplification within the transgenome (Figure 1a). Understanding the nature and extent of these CMGT associated chromosomal rearrangements is important to the overall assessment and exploitation of the CMGT process. Long-range restriction mapping by pulsed-field gel electrophoretic analysis (19) can address this question and is in progress.

Our results add to the molecular definition of the WAGR locus. Mapping of the E65-6 derived markers to the WAGR deletion and associated translocation hybrids defines seven discrete intervals which subtend band 11p13. Two of the markers, λE65-6-10B and λE65-6-74, map to a new interval which is centromere distal to the aniridia (SMX) and the Potter facies (POR) associated translocation breakpoints, but centromere proximal to FSHB. The smallest region of overlap for the WAGR locus is redefined as lying between the centromere proximal breakpoint in ANX6.14 and the centromere distal breakpoint in SAX3.10. The close proximity of the Wilms' tumour (WT) and aniridia (AN2) loci is confirmed and refined. Similarly, the physical location of the POR

translocation breakpoint within the smallest region of overlap for WAGR is maintained and, therefore, the possibility of a genetic relationship between genitourinary dysplasia and Wilms' tumour is strengthened (3).

With the density of markers now achieved for this region, it should be possible to construct a long-range restriction map of normal and WAGR associated chromosomes 11 by pulsed-field gel electrophoretic analysis (19) and thus define precisely the molecular size of the SRO and the positions of the POR and SMX translocation breakpoints. An even higher density of markers is available for the 11p15.4 to 11pter region. A precise physical map of this region would permit comparison with the detailed genetic map already available (20) and a direct route to disease genes mapping in this region (21).

ACKNOWLEDGEMENTS

We thank Hazel Cameron, Anne Seawright and Judy Fletcher for technical assistance.

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